PHOTOSYNTHETIC STUDIES ON EIGHT FILMY FERNS (HYMENOPHYLLACEAE) FROM SHADED HABITATS IN MALAYSIA

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ABSTRACT

The photosynthetic characteristics of eight Malaysian Hymenophyllaceae filmy ferns from shady habitats were investigated in this study. Chlorophyll content was highest in *Trichomanes meifolium*, followed by *Cephalomanes obscurum, Hymenophyllum serrulatum, H. denticulatum, H. javanicum, H. acanthoides, H. exsertum* and *H. blandum* with values ranging from 3.3 to 8.6 mg g^{-1} fresh weight, compared to two sun ferns, *Dicranopteris linearis* and *Nephrolepis biserrata.* Soluble protein content was remarkably high in *H. serrulatum,* followed by *Dicranopteris linearis* and *Nephrolepis biserrata.* Protein to chlorophyll ratio in the filmy ferns was low compared to the sun ferns. Chloroplast number and size ranged between 34 to 138 per cell profile and between 4.8µm to 6.5µm in diameter, in the Hymenophyllaceae. Rubisco RbcL profiling showed clear differences between the filmy ferns and the sun species. Quantum efficiency measurements in four Hymenophyllaceae spp exhibited Fv/Fm values ranging between 0.73 to 0.81. The Hymenophyllaceae species also showed low *in vivo* CO_2 assimilatory rates and light saturation points, ranging between 5 to 17 µmol CO_2 m^{-2} s^{-1} and between 100 to 150 µmol m^{-2} s^{-1} respectively, except for *H. blandum* which saturated at around 300-400 µmole $m^{-2}s^{-1}$. In contrast, the sun ferns *Dicranopteris linearis* and *Nephrolepis biserrata*, showed higher CO_2 saturation rates, 22 µmol CO_2 m^{-2} s^{-1} and 30 µmol CO_2 m^{-2} s^{-1}, respectively, and higher light saturation point at around ~600µmole $m^{-2}s^{-1}$.The findings add further to our understanding on how the filmy ferns adapt and thrive in their humid and shady habitats.

ABSTRAK

Perbandingan ciri-ciri fotosintesis ke atas lapan spesies tumbuhan Hymenophyllaceae dari habitat teduh telah dijalankan dalam kajian ini. Kajian telah menunjukkan bahawa kandungan klorofil adalah lebih tinggi dalam *Trichomanes meifolium*, diikuti oleh *Cephalomanes obscurum*, *Hymenophyllum serrulatum, H. denticulatum, H. javanicum, H. acanthoides, H. exsertum* dan *H. blandum* dengan nilai antara 3.3-8.6 mg g^{-1} berat segar, berbanding dengan dua spesies paku-pakis dari habitat terbuka, *Dicranopteris linearis* dan *Nephrolepis biserrata*. Kandungan protein terlarut pula adalah amat tinggi dalam *H. serrulatum*, diikuti oleh *Dicronopteris linearis* dan *Nephrolepis biserrata.* Nisbah protein terlarut / klorofil dalam spesies Hymenophyllaceae adalah lebih rendah berbanding dengan paku-pakis dari habitat suria. Bilangan kloroplas dan saiz kloroplas dalam *Hyemnophyllaceae* adalah di antara 34 hingga 138 per sel dan di antara 4.8 μm hingga 6.5 μm diameter. Selain itu, profil protein rubisco dalam tumbuhan teduh Hymenophyllaceae dan paku-pakis dari habitat terbuka juga turut menunjukkan perbezaan yang jelas. Nilai ukuran kecekapan kuantum dalam lapan jenis tumbuhan Hymenophyllaceae menunjukkan nilai Fv/Fm adalah antara 0.73 hingga 0.81. Tumbuhan Hymenophyllaceae juga menunjukkan nilai kadar fotosintesis yang rendah, iaitu di antara 5 hingga 17 μmol CO_2 m^{-2} s^{-1} dan kadar ketepuan cahaya matahari adalah di antara 100 hinga 150 μmol m^{-2} s^{-1}, kecuali bagi *H. blandum* di mana kadar ketepuan cahaya matahari adalah di antara 300 ke 400 μmol m^{-2} s^{-1}. Manakala, *Dicranopteris linearis* dan *Nephrolepis biserrata* menunjukkan nilai kadar fotosintesis yang tinggi, iaitu di antara 22 hingga 30 μmol CO_2 m^{-2} s^{-1} dan kadar ketepuan cahaya matahari adalah pada ~600 μmol m^{-2} s^{-1}. Hasil daripada kajian ini menambahkan lagi pemahaman kita tentang bagaimana tumbuhan teduh ini menyesuaikan diri dan hidup di dalam habitat yang lembap dan teduh.

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TABLE OF CONTENT

			Page
Title			**i**
Original Literary Work Declaration			**ii**
Abstract			**iii**
Abstrak			**iv**
Acknowledgments			**v**
Table of contents			**vi**
List of Figures			**viii**
List of Tables			**xi**
List of Abbreviations			**xiii**
Chapter 1	**:**	**Introduction**	**1**
1.1		**Hypothesis**	**3**
1.2		**Objectives of study**	
Chapter 2	**:**	**Literature Review**	**4**
2.1		**General Introduction**	
2.2		**Characteristic of Plants from Habitats of Different Irradiances**	
2.3		**Adaptation of plant to growth under Different Light regimes**	
2.5		**Filmy ferns**	
Chapter 3	**:**	**Methodology**	**33**
3.1		**Choice of plant materials**	
3.2		**The site chosen for study and collection**	
3.3		**Chlorophyll determination**	
3.4		**Protein analysis**	
3.5		**Measurement of light intensity, relative humidity and temperature**	
3.6		**Chlorophyll fluorescent measurement**	
3.7		**Gas exchange and photosynthetic rate measurement**	
3.8		**Microscopy**	
3.9		**Plant protein extraction**	
3.10		**SDS-PAGE and Rubisco expression detection**	
3.11		**Statistic analysis**	

Chapter 4	**:**	**Results**	**43**
4.1		**Environmental measurement of the Habitats from which the specimens were Collected**	
4.2		**Leaf chlorophyll content of *Hymenophyllaceae* Species and two sun ferns**	
4.3		**Leaf soluble protein content of *Hymenophyllaceae* and two sun ferns Determined via Lowry, Bradford and BCA Methods**	
4.4		**Mesophyll chlorophyll number in the leaves of *Hymenophyllaceae* Species**	
4.5		**Chlorophyll Fluorescence and Photosynthetic Light Response Curves in the leaves of *Hymenophyllaceae* species collected**	
4.6		**Protein expression in *Hymenophyllaceae* and two sun ferns leaves**	
Chapter 5	**:**	**Discussion**	**66**
Chapter 6	**:**	**Conclusions**	**78**
References			**80**
Appendices			**90**

List of Figures

	Page
Figure 1 : Electron micrographs of a chloroplast from *Alocasia macrorrhiza* at low irradiance,10 µmole photons $m^{-2}s^{-1}$. (Adapted from Chow *et al.,* 1988)	16
Figure 2 : Large grana each containing over 110 thylakoids in upper epidermal cell plastids of *Teratophyllum rotundifoliatum* (Adapted from from Nasrulhaq-Boyce & Duckett,1991)	16
Figure 3 : Light micrographs of the crosssections of (a) *P. cirratum* subsp. *macrophyllum* leaf, (b) *P. subtortile* leaf showing 2-4 cells high and *Pogonatum neesii* leaf showing 5-7 cells high. (Adapted from Nasrulha-Boyce *et al.,* 2011)	17
Figure 4-6: *Hymenophyllum acanthoides*	23
Figure 7 : *Hymenophyllum blandum*	24
Figure 8 : *Hymenophyllum javanicum*	25
Figure 9-11 : *Hymenophyllum exsertum*	25-26
Figure 12 : *Hymenophyllum serrulatum*	27
Figure 13 : *Hymenophyllum denticulatum*	27
Figure 14-16: *Trichomanes meiofolium* on a rotting log at Genting Highlands	28-29
Figure 17 : *Cephalomanes obscurum* found under rock ledges.	30
Figure 18 : *Dicranopteris linearis* densely covering a roadside of the lowlands	31
Figure 19 : *Nephrolepis biserrata* growing in the lowlands.	32
Figure 20: Map of Gunung Ulu Kali, Genting Highlands, Pahang, Malaysia, where the ferns were collected from 42	33
Figure 21 : Light micrographs showing chloroplasts in leaf cells of *H. serrulatum*, 40x	51
Figure 22: Light micrographs showing chloroplasts in leaf cells of *H. javanicum*, 40x	51
Figure 23: Light micrographs showing chloroplasts in leaf cells of *H. acanthoides,* 40x	52

Figure 24: Light micrographs showing chloroplasts in leaf cells of *H. exsertum*, 40x 52

Figure 25: Light micrographs showing chloroplasts in leaf cells of *H. blandum*, 40x 53

Figure 26: Light micrographs showing chloroplasts in leaf cells of *Hymenophylllum denticulatum*, 40x 53

Figure 27: Light micrographs showing chloroplasts in leaf cells of *Cephalomanes obscurum*, 120x 54

Figure 28: The effect of varying light intensity on the photosynthetic rates in the leaves of *Trichomanes meiofoloium.* Points show the mean values calculated from 3 readings. 58

Figure 29: The effect of varying light intensity on the photosynthetic rates in the leaves of *Hymenophyllum javanicum.* Points show the mean values calculated from 3 readings 58

Figure 30: The effect of varying light intensity on the photosynthetic rates in the leaves of *Hymenophyllum denticulatum.* Points show the mean values calculated from 3 reading 59

Figure 31: The effect of varying light intensity on the photosynthetic rates in the leaves of *Hymenophyllum exsertum.* Points show the mean values calculated from 3 readings 59

Figure 32: The effect of varying light intensity on the photosynthetic rates in the leaves of *Hymenophyllum serrulatum.* Points show the mean values calculated from 3 readings 60

Figure 33: The effect of varying light intensity on the photosynthetic rates in the leaves of *Hymenophyllum acanthoides.* Points show the mean values calculated from 3 readings 60

Figure 34: The effect of varying light intensity on the photosynthetic rates in the leaves of *Hymenophyllum blandum.* Points show the mean values calculated from 3 readings 61

Figure 35: The effect of varying light intensity on the photosynthetic rates in the leaves of *Cephalomanes obscorum.* Points show the mean values calculated from 3 readings 61

Figure 36: Photosynthetic light response curves of common Malaysian sun fern species, *Dicranopteris linearis* . Each point is an average of 3 readings 62

Figure 37: Photosynthetic light response curves of common Malaysian sun fern species, *Nephrolepis biserrata.* Each point is an average of 3 readings 62

Figure 38 : SDS gel electrophoresis of soluble protein from the leaves of two sun ferns and four species of *Hymenophyllaceae* 64

Figure 39: Immunodetection of Rubisco protein in two sun ferns and four *Hymenophyllaceae* species 65

List of Tables

	Page
Table 1: Characteristic differences between plants adapted or acclimated to sunny *v*. shade extremes	5
Table 2: Chlorophyll content per fresh weight/leaf area and chlorophyll *a* and chlorophyll *b* ratio	9-10
Table 3: Chlorophyll content and chlorophyll *a:b* ratio for fern species (Unpublished data from Nasrulhaq-Boyce)	10-11
Table 4: Soluble protein content and ratio of soluble protein /chlorophyll content for fern species	13-14
Table 5: List of species that were collected from Gunung Ulu Kali, Pahang.	33
Table 6: A summary of environmental measurements in the habitats from which the species were collected	44
Table 7: Leaf chlorophyll content of Hymenophyllaceae species and two sun ferns.	46
Table 8: Leaf soluble protein content of Hymenophyllaceae species and two sun ferns determined via the Lowry, Bradford and BCA methods	48
Table 9: Mesophyll chloroplast number per profile in the leaves of the Hymenophyllaceae species.	50
Table 10: Chlorophyll fluorescence in the leaves of the Hymenophyllaceae species collected.	57
Table 11: Total chl, chl *a,* chl *b*, chl *a/b* content Of *Hymenophyllaceae* Species And Sun Ferns	88
Table 12: Protein Content (Lowry Assay) Of *Hymenophyllaceae* Species & Sun Ferns	92
Table 13: Protein Content (Bradford Assay) Of *Hymenophyllaceae* Species & Sun Ferns	95
Table 14: Protein Content (BCA Assay) Of *Hymenophyllaceae* Species & Sun Ferns	98
Table 15: BSA Standard Curve (Bradford Assay)	101
Table 16: BSA Standard Curve (BCA Assay)	102

Table 17: BSA standard curve (Lowry assay) 103

Table 18: Chloroplast number per cell profile of eight *Hymenophyllaceae* species 104

Table 19: Chloroplast size (μm) of eight *Hymenophyllaceae* species 105

Table 20: Chlorophyll fluorescence in four Species of *Hymenophyllaceae* 108

Table 21: Photosynthetic rates of eight *Hymenophyllaceae* species 109-112

Table 22: Photosynthetic rates of two sun ferns species 113

List of Abbreviations

Abs	Absorbance
et al.	et alia (and others)
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
cm	centimeter
chl	Chlorophyll
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CO_2	Carbon dioxide
$CuSO_4$	Copper(II) sulfate
dH_2O	Distilled water
ddH_2O	Double distilled water
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Fm	Maximum fluorescence yield
Fo	Minimum fluorescence yield
Fv/Fm	Optimum quantum yield (Fv=Fm-Fo)
FW	Fresh weight
G	Gram
HCL	Hydrochloric acid
kDa	Kilo Dalton
KCl	Potassium chloride
L	Liter
LED	Light-emitting diode

M	metre
M	molar
Mg	milligram
Na_2CO_3	Sodium carbonate
NaOH	Sodium Hydroxide
µg	microgram
µmole	micromoles
ml	milliliter
mm	millimeter
O_2	Oxygen
PAR	Photosynthetic active radiation
PSII	Photosystem II
R	Replicates
RT	room temperature
rpm	Revolutions per minute
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase,
s	Second
SE	Standard error
SD	Standard Deviation
TCA	Trichloroacetic acid
USA	United States of America
UK	United Kingdom
v	versus

CHAPTER 1

INTRODUCTION

The energy captured by the plants via photosynthesis provides green plants with almost all of their chemical energy and is central to their ability to compete and reproduce. In turn the photosynthetic process is directly and dramatically influenced by the amount of light striking a plant's leaves. Thus plants have different photosynthetic characteristics when grown under different light intensities. Plants growing under different light intensities can considerably differ in their relative composition of photosynthetic pigments, electron carriers, chloroplast ultrastructure and photosynthetic rates. It also has been well acknowledged in the literature that these plants, particularly in the angiosperms, exhibit striking differences in their morphology, ultrastructure, physiology and biochemistry (Boardman, 1977; Lee *et al*., 1990). Generally, the maximum photosynthetic rate, at optimal temperature and under conditions of light saturation, will be higher in plants grown under high light intensity than plants grown under low light intensity. Many researchers have studied how different levels of photosynthetic active radiation affect photosynthesis and how the leaf traits that develop under different levels of irradiances influence a plant's photosynthetic response to light level (Nasrulhaq-Boyce & Mohamed, 1987; Nasrulhaq-Boyce & Duckett, 1991; Proctor, 2003; Marschall & Proctor, 2004). Thus, comparative studies of the photosynthetic response and leaf characterisitics of plants growing under different levels of irradiances have provided crucial insights into the significance of several leaf traits observed in plants adapted to different light conditions in their natural habitat. Species, ecotypes or acclimated forms with higher rates of leaf photosynthesis under specific

light levels have been inferred to have an edge in energy capture and competitive ability under sunny and shady conditions.

Thus, in order to understand further how low-light plants thrive and survive in shady environments and to see how different plants, particularly the ferns, adapt and thrive in their chosen shaded habitats, for this study the Hymenophyllaceae group of shaded ferns was chosen for investigation. These filmy ferns (*Hymenophyllaceae)* are attractive and live most abundantly in the humid tropical rainforest. They show an amazing diversity in terms of their morphology and the habitats they occupy, making them a good model for studying the ecology and related adaptive survival strategies in pteridophytes (Dubuisson *et al,* 2003). Their physiology has received little attention despite the large amount of literatures on their morphology and taxonomy. Fairly recently Proctor (2003) studied the comparison of ecophysiological measurements on the light response, water relations and dessication tolerance of the filmy ferns, *Hymenophyllum wilsonii* and *Hymenopyllum tunbrigense.* He reported that both the filmy fern species were well adapted to a low light environment, where *H. willsoni* has higher light requirement than *H. tumbrigense.* Earlier, Johnson (2000) working on adaptations of extreme low lights of *Trichomanes speciosum* reported on the similarities among *Hymenophyllaceae* species in terms of ecological adaptation.

The aim of this study was to evaluate the chlorophyll, protein content and chloroplast anatomy in selected *Hymenophyllaceae* as well as their photosynthetic activity and protein profiles (Rubisco expression). In addition chlorophyll fluorescence was investigated together with Rubisco expression, in an attempt to understand further photosynthesis in shaded ferns. It is hoped that the results will provide a better understanding on how plants thrive and adapt to living in shady environments.

1.1 Hypothesis

The *Hymenophyllaceae* ferns growing in shady habitats would demonstrate a high level of chlorophyll content, larger chloroplasts, low levels of soluble protein content, low levels of Rubisco expression, in addition to low photosynthetic rates and low photosynthetic light saturation rates compared to ferns living in high light intensities.

1.2 Objectives of Studies

The main objective of this research was to study the photosynthetic characteristics of selected Hymenophyllaceae ferns species and to see how it relates to how they thrive and adapt in their respective habitats. Hence, the following parameters were investigated in the selected Hymenophyllaceae species;

i. Chlorophyll *a* and *b* content, Chlorophyll *a*/*b* ratio and total chlorophyll content.
ii. Protein content and protein/chlorophyll ratio.
iii. Photosynthetic quantum efficiency (Fv/Fm).
iv. Photosynthetic carbon dioxide assimilation rate and light response curves.
v. Rubisco protein expression.

CHAPTER 2

LITERATURE REVIEW

2.1 General Introduction

Studies on plants from habitats of different light intensities, especially on angiosperms have been carried out for many years, starting from the early 1970s. It has been well documented that plants from habitat of low irradiance are incapable producing high photosynthetic rates, although they perform efficiently at low light irradiances (Boardman, 1977; Givnish, 1988). Plants exhibit obvious differences in their morphology, ultrastructure, physiology and biochemistry depending on the light intensities they have been exposed to in their habitat or under experimental conditions (Table 1). Plants that grow under lower irradiances possess fewer, larger chloroplast with larger grana and higher pigment content, including lower photosynthetic rates. For most plants that grow in high light intensities in their natural habitats, show contrasting characteristics than do their shade counterparts, with higher rates of photosynthesis at saturating light intensity for example. Similar but comparatively fewer studies have also been reported in ferns and bryophytes (Nasrulhaq-Boyce & Mohamed, 1987; Nasrulhaq-Boyce & Duckett, 1991; Proctor, 2003; Marschall & Proctor, 2004). A study on four types of Malaysian ferns by Nasrulhaq-Boyce and Mohamed (1987) showed that the shade ferns exhibted a greater chlorophyll content, and lower protein, protoheam content than do in sun ferns. They concluded shade ferns have physiological characteristic favouring lower capacity for photosynthesis due to their shady habitats. Similar studies on temperate ferns from habitats of different irradiances have also shown that the chlorophyll content was greater in the shade ferns when compared to the ferns living in open exposed area. (Hew & Wong, 1974; Ludlow & Wolf, 1975). They

reported that the sun ferns had a higher stomatal density and showed greater capacity for *in vivo* photosynthesis and respiration than their shade counterparts. Table 1 shows comparative differences between sun and shade plants derived from Givnish (1988).

Table 1: Characteristic differences between plants adapted or acclimated to sunny *v.* shade extremes

Trait	Sun	Shade
(1) Leaf-level		
Photosynthetic light response		
Light-saturated rate	High	Low
Compensation irradiance	High	Low
Saturation irradiance	High	Low
Biochemistry		
N,Rubisco and soluble protein content/mass	High	Slightly lower
Chlorophyll a/chlorophyll b ratio	High	Low
Chlorophyll/soluble protein ratio	Low	High
Anatomy and ultrastructure		
Chloroplast size	Small	Large
Thylakoid/ grana ratio	Low	High
Morphology		
Leaf mass/area	High	Low
Leaf thickness	High	Low
Stomatal size	Small	Large
Stomatal density	High	Low
Palisade/spongy mesophyll ratio	High	Low
Mesophyll cell surface/leaf area ratio	High	Low
Leaf orientation	Erect	Horizontal
Iridescence, lens-shaped epidermal cells	None	Rare
Raddish leaf undersides	Very rare	Infrequent
(2) Canopy –level		
Leaf area index	High to low	Low
Phyllotaxis	Spiral	Distichous
Twig orientation	Erect	Horizontal
Assymmetric leaf bases	Very rare	Infrequent
(3) Plant-level		
Fractional allocation to leaves	Low	High
Fractional allocation to roots	High	Low
Reproductive effort	High	Low

Derived from Givnish (1988)

Besides shade ferns, shade mosses also exhibit a similar photosynthetic characteristics, for example having greater chlorophyll content than mosses living in sunny environments. Recently, Nasrulhaq-Boyce *et al.* (2011) studied the morphology

and photosynthetic rates on three species of mosses (*Pogonatum)* from habitats of varying light irradiances in Malaysia. They reported that the shade *Pogonatum cirratum* subsp. *macrophyllum* exhibited lower photosynthetic light saturation levels than their sunnier relatives, *P. neesii* and *P. subtortile*. They observed that *in vitro* Photosystem II photochemical rates and CO_2 assimilatory rates were highest in the sun *Pogonatum neesii*, even at low light intensities. They also reported a higher soluble protein content in the sun species compared to its shade counterparts, suggesting this was probably due to the presence of higher amounts of ribulose bisphosphate carboxylase oxygenase (Rubisco) in the leaves.

Extreme shade plants can survive much lower light intensities than do sun plants. Studies have also shown that sun plants, when grown under low light intensities, show light saturation curves that almost resemble those of shade plants (Bjorkman, 1967). Hence, classification of plants into sun and shade species cannot be made based on the basis of light saturation curves alone. They can be classified depending on their preference for living in habitats with a particular irradiance. In fact true shade plants not only survive but thrive at low irradiance than the species that live in full sunlight.

2.2 Characteristic Of Plants From Habitats Of Different Irradiances

2.2.1 Leaf Morphology, Position and Orientation

Leaves have the ability to adapt, morphologically as well as physiologically, to the light intensities present in their habitat. Thus, plants that grow in shady habitats have different characteristics in terms of morphology, anatomicals, structures and physiological activities when compared to those growing in full sunlight. Generally plants growing under full sunlight possess thicker smaller leaves than their shady counterparts. The thinner and larger leaves of the shade species is believed to help facilitate the passage of the lower intensity light through the leaves to reach the

photosynthetic machinery in the chloroplasts. However thin leaves is not always characteristic of shady leaves. Many species have thick leaves and high chlorophyll content per unit leaf area contradictory to the general rule. A comparison was made between *Atriplex patula* plants grown under three different light intensities and it was found that those grown under high light intensity had thicker leaves and more chloroplast per cell (Boardman, 1977).

Studies on sun and shade *Prunus serotina*, an angiosperm species have shown that sun leaves have a greater thickness, specific mass, area and stomatal density and lower guard cell length than shade leaves (Abrams *et al.,* 1992). These morphological characteristics explained the ability of sun and shade plants to survive in greatly contrasting environments. With regard to pteridophytes, Brach *et al.* (1993) have studied the morphological characteristics of ferns growing under contrasting light intensities. They reported that sun-grown ferns had significantly higher dry mass per unit leaf area than shade-grown ferns, and they observed that the sun leaves of sun-loving ferns were yellow green in color as compared to darker green shade leaves of shade-loving ferns.

A prime factor governing a leaf's photosynthetic productivity is its position in the canopy, which determines its light environment and its rate of net CO_2 uptake. The leaf arrangement, type of leaves and the total area of a plant usually promote maximum utilization of available light. For example, it was shown that in the beech (*Fagus sylvatica*), the sun leaves orientate at an acute angle to the plane illumination and are found towards the crown. Beneath them are the shade leaves which are at right angles to the direction of illumination to ensure maximum interception of light (Fogg, 1976). The vertical orientation of the upperleaves present large gaps between them, allowing flecks of sunlight to reach the foliage lower down. The plants growing under a thick forest canopy are exposed to light containing a relative abundance of green wavelenghts but

limited in the blue and red colors. The discrepancy between the energy and quantum fluxes on the forest floor is most probably due to the selective absorption of photosynthetically active wavelenghts by the leaf canopy. This will almost surely decrease the ability of the shorter plants to carry out maximum photosynthesis. However, these shorter shade plants perform efficiently at low light intensities, although they are incapable of high photosynthetic rates (Boardman, 1977).

2.2.2 Chlorophyll content

It is well documented in the literature that plants from habitats of low irradiance are richer in chlorophyll than the leaves of plants that live in open spaces. They generally contain a higher amount of chlorophyll, particularly a higher proportion of chlorophyll *b* relative to chlorophyll *a* (ratio chlorophyll *a/b* is low). This characteristic has been observed in sun and shade plants of many species, as well as when a single species is grown under different light intensities (Boardman, 1977; Givnish, 1988). The higher content of chlorophyll *b*, which is closely associated with photosystem 2 and the granum, in shade plants is believed to increase the light absorbing capacity of the shade leaves as the wavelength of light that reach the forest floor is different from that at the canopy. Although in their native habitats, shady leaves often have a higher total chlorophyll content on a fresh weight basis than do sun leaves, the chlorophyll content on a unit leaf area is usually lower in shady leaves so they are able to make more efficient use of small amounts of light. However some shade species such as *Cordyline rubra* and *Lomandra longifolia* have thick leaves, a high ratio of dry matter to leaf area and a high chlorophyll content per unit area of leaf (Boardman, 1977).

Recent studies have shown a higher chlorophyll content expressed on a fresh weight basis in the shade moss *Pogonatum cirratum* compared to its more sunlit cousins (Nasrulhaq-Boyce *et al.*, 2011). Similar chlorophyll contents have also been reported in the ferns *Selaginella wildenowii* (Hebant & Lee, 1984) and also in the deep shade fern

Teratophyllum rotundifoliatum (Nasrulhaq-Boyce & Duckett, 1991). The high chlorophyll content in the shade plant leaves is probably an adaptation for the plant to capture all available light quanta that reaches the leaves on the floor of the forest. Similar studies also has been reported in other nation-wide ferns species (Matthew *et al*., 2005; Alfredo *et al*., 2010). Table 2 shows comparative differences of chlorophyll content between sun and shade plants derived from Boardman (1977), Nasrulhaq-Boyce & Mohamed (1987) and Huang *et al.* (2011).

Table 2 : Chlorophyll content per fresh weight/leaf area and chlorophyll *a* and chlorophyll *b* ratio

Fern species	Chlorophyll content per fresh weight (mg/g)	Chlorophyll content per leaf area (mg/dm^2)	Ratio chlorophyll *a*:*b*	References
Shade				Boardman (1977)
Adenocaulon bicolor	3.2	3.2	-	
Aralia californica	3.0	2.6	-	
Disporum smithii	2.8	2.5	-	
Trillium ovatum	3.4	4.0	-	
Alocasia macrorrhiza	-	5.8	-	
Cordyline rubra	-	8.7	-	
Lomandra longifolia	-	12.2	-	
Pseudocarpia nitidula	-	6.5	-	
Sun			-	
Atriplex patula	1.8	4.0		
Echinodorus berteroi	2.3	4.6	-	
Mimulus cardinalis	1.6	5.2	-	
Plantago lanceolata	2.2	5.3	-	
Solidago spathulata	1.8	4.3	-	
Shade				Nasrulhaq-Boyce &
Abacopteris multilineata	2.10 ± 0.22	3.1	2.4	

Christensenia aesculifolia	2.55 ± 0.23	9.9	2.6	Mohamed (1987)
Tectaria singaporeana	2.40 ± 0.10	3.1	2.1	
Tectaria vasta	2.05 ± 0.24	3.0	2.2	
Sun				
Blechnum orientale	1.90 ± 0.08	4.0	2.8	
Dicranopteris linearis	1.49 ± 0.21	2.6	2.6	
Lygodium scandens	1.55 ± 0.16	2.5	2.9	
Stenochlaena palustris	1.79 ± 0.01	3.4	2.5	
Shade				
Athyrium pachyphlebium	2.47 ± 0.04	-	1.56 ± 0.06	Huang. D *et al.* (2011)
Sun				
Athyrium pachphlebium	1.78 ± 0.02	-	1.8 ± 0.04	

Shady leaves have more chlorophyll, especially chlorophyll *b*, as it might be expected, from their enhanced membrane content (Anderson *et al*, 1988; Salisbury & Ros, 1991). Studies by Anderson *et al* (1988) have shown that the shade plant thylakoids have chlorophyll *a:b* ratios of 2.0-2.2 compared to 2.6-3.6 for sun plant thylakoids. Anderson *et al.* (1988) indicated that a lower *a/b* ratio in shade plants reflects an increment in light harvesting complex 2 (LHC2) complexes relative to reaction centres. This has been shown to hold true across the whole group of fern plants, as shown in Table 3 (Unpublished data from Nasrulhaq-Boyce).

Table 3: Chlorophyll content and chlorophyll *a:b* ratio for fern species (Unpublished data from Nasrulhaq-Boyce)

Fern species	Chlorophyll content per fresh weight (mg/g)	Chlorophyll content per leaf area (mg/dm^2)	Ratio chlorophyll *a:b*
Shade			
Abacopteris multilineata	2.1	3.1	-

Christensenia aesculifolia	2.55	9.9	-
Tectaria singaporeana	2.4	3.1	-
Tectaria vasta	2.05	3.0	-
Teratophyllum rotundifoliatum	6.13 ± 0.94	6.1 ± 0.7	1.32 ± 0.04
Asplenium nidus	1.814 ± 0.16	0.06 ± 0.015	1.63 ± 0.23
Cyathea latebrosa	-	0.0175 ± 0.004	1.65 ± 0.23
Davalia denticulata	3.59 ± 0.18	0.07 ± 0.008	0.734
Sellaginella plana	4.63 ± 0.39	-	1.52 ± 0.17
Sun			
Blechnum orientale	1.9	4.0	-
Dicranopteris linearis	1.49	2.6	-
Lygodium scandens	1.5	2.5	-
Stenochlaena palustris	1.79	3.4	-
Drymoglossum piloselloides	1.07 ± 0.21	-	0.84 ± 0.03
*Asplenium nidus**	1.067 ± 0.29	0.032 ± 0.011	1.72 ± 0.367
*Cyathea latebrosa**	-	1.6 ± 0.22	1.52 ± 0.06
*Davalia denticulata**	1.84 ± 0.1	0.05 ± 0.01	0.628
Cyathea contaminans	1.17 ± 0.33	-	1.4 ± 0.33

***Species that can live in both sun and shade environment**.

2.2.3 Soluble Protein Content

Irrespective of a higher total chlorophyll content on a fresh weight basis, shade leaves have been shown to have lower soluble protein content and considerably lower ratios of soluble protein to chlorophyll than do the sun species. The low content of soluble protein in shade plant leaves are also reflected in the lower activity of ribulose bisphosphate carboxylase (Rubisco) which is a major soluble protein in leaves (Givnish, 1988; Nasrulhaq-Boyce & Mohamed, 1987). This is possibly the reason for the high light saturated photosynthetic rates in sun plants (Boardman, 1977; Nasrulhaq-Boyce & Mohamed, 1987). Analysis by Bjorkman (1981) indicated that the low levels of Rubisco in shade plants are adaptive as Rubisco content is correlated with rates of dark respiration, and thus inversely correlated with net photosynthesis at low irradiance levels.

In general, Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the most abundant soluble protein in leaves and makes up 50% or more of all protein in the biosphere. Rubisco is the main enzyme assimilating carbon dioxide into the biosphere which then becomes incorporated into carbohydrates usable for most living organisms. Rubisco is made up of eight small (~14 kDa) and eight large (~56 kDa) subunits arranged as eight heterodimers (Malkin & Niyogi, 2000). At low light intensity, where the capacity for RuBP regeneration typically limits photosynthesis, the efficiency of Rubisco use is potentially low, as evidenced by the fact that the activity of the enzyme is generally reduced by these regulatory mechanisms to match the reduced capacity for RuBP regeneration (Kobza & Seemann, 1988). Plants which grow at low light intensity might be expected to produce less Rubisco per unit leaf area than plants growing at high light intensity to regulate its activity in such a way that it is fully active at lower light intensity.

Studies on Malaysian sun and shade ferns have shown that sun-type fern expressed higher ratio soluble protein/ chlorophyll, ranging from 40 mg $mg^{-1}$to 62 mg mg^{-1}, compared with the shade-type ferns, which ranged from 9 mg $mg^{-1}$to 22 mg mg^{-1} (Nasrulhaq-Boyce & Mohamed, 1987). Boardman (1988) showed that the shade plant have soluble protein/chlorophyll ratios of ~2.8-3.3 mg mg^{-1} compared to ~11.1 mg mg^{-1} for sun plant thylakoids. Studies on the deeply shaded *Teratophyllum rotundifoliatum* and *Teratophyllum aculeatum*, also showed lower soluble protein content (36.3- 45.2 mg g^{-2}) and lower soluble protein to total chlorophyll ratio, 6.72-7.35 mg mg^{-2}. Results from other studies with ferns are shown in Table 4.

Table 4 : Soluble protein content and ratio of soluble protein /chlorophyll content for fern species

Fern species	Soluble protein contetn (mg g^{-1}fresh weight)	Ratio Protein/ Chlorophyll (mg g^{-1} chl)	References
Shade			
Adenocaulan bicolor	-	3.1	Boardman (1977)
Aralia californa	-	4.8	
Disporum smithii	-	2.0	
Trillium ovatum	-	3.3	
Alocasia macrorrhiza	-	2.8	
Cordyline rubra	-	2.0	
Lomandra longifolia	-	2.8	
Pseudocarpa nitidula	-	3.7	
Abacopteris multimineata	-	16.7	Nasrulhaq-Boyce &
Christensenia aesculifolia	-	9.0	Mohamed (1987)
Tectaria singaporeana	-	13.1	
Tectaria vasta	-	21.9	
Teratophyllum rotundifoliatum	45.2 ± 6.0	7.35 ± 2.9	Unpublished data
Teratophyllum aculeatum	36.3 ± 2.9	6.72 ± 0.54	
Davalia denticulata	3.25 ± 0.02	0.91	Unpublished data

Cyathea latebrosa	5.76 ± 1.15	0.45	Unpublished data
Sellaginella plana	2.08 ± 0.02	2.22	Unpublished data
Asplenium nidus	3.45 ± 0.02	1.90	Unpublished data
Sun			
Atriplex patula	-	13.0	Boradman (1977)
Echinodorus berteroi	-	13.8	
Mimulus cardinalis	-	9.6	
Plantago lanceolata	-	7.2	
Solidago spathulata	-	11.8	
Blechnum orientale	-	62.1	Nasrulhaq-Boyce & Mohamed (1987)
Dicranopteris linearis	-	40.3	
Lygodium scandens	-	69.7	
Stenochlaena palustris	-	59.2	
Drymoglossum piloselloides	8.63 ± 2.30	8.07	Unpublished data
Davalia denticulata	3.94 ± 0.03	2.14	Unpublished data
Cyathea latebrosa	3.77 ± 0.42	0.39	Unpublished data
Cyathea contaminans	7.13 ± 0.66	6.09	Unpublished data
Asplenium nidus	4.00 ± 0.02	3.75	Unpublished data

2.2.4 Chloroplast Structure

It is well documented in previous literature that full sun and extreme shade leaves exhibit striking contrast in their anatomy, ultrastructure, physiology and biochemistry (Boardman,1977; Givnish,1988; Lee *et al.*,1990). In order to cope with the

lower quantity and quality of light reaching the shady habitats, shade plants possess fewer, larger chloroplast with larger grana. Chloroplast is an important organelle in the photosynthetic process and thus different light intensity can affect its structure, number and size . Light intensity plays an important role in the development of chloroplast in almost all plant species. Recent studies have shown that, leaves of plants living in the low light irradiance generally have fewer chloroplasts per cell but they are larger in size compared to leaves of plants that live in high light irradiance (Duckett & Nasrulhaq-Boyce, 1991). These striking features of shade plants may contain as many as 100 thylakoids per granum (Nasrulhaq-Boyce & Duckett, 1991). Previous literature have reported that the grana are irregularly arranged within a chloroplast and not orriented in one places as they are in sun plant chloroplast (Boardman,1977) . It is believed that this orientation in the shade plant chloroplasts might be expected to improve their performance for the collection of the weak diffuse radiation on the forest floor.

Anderson *et al.* (1988) indicated that a lower *a/b* ratio in shade plants reflects an increment in light harvesting complex 2 (LHC2) complexes relative to reaction centres. Chlorophyll *a* and *b* are both associated with the light harvesting antennae, although more chlorophyll *b* is found in photosystem 2 . It is believed that the LHC2 are involved in thylakoid appression and formation of granal stacks; since the thylakoid membranes in shady plants are much higher. It also contributes to the increment of photosyntetic system II (PSII) antenna content (LHC2). Chow *et al.* (1988) reported a large number of thylakoids in the high iradiance-adapted *Alocasia macrorrhiza* when grown in shaded environment (Figure 1). Nasrulhaq-Boyce and Duckett (1991) reported the largest grana for land plants in the deep shade fern *Teratophyllumn rotundifoliatum.* They also have reported the epidermal chloroplast in *Teratophyllum rotundifoliatum* contain numerous large grana, with no preferred orientation, and integranal lamellae completely filling the stroma (Figure 2).

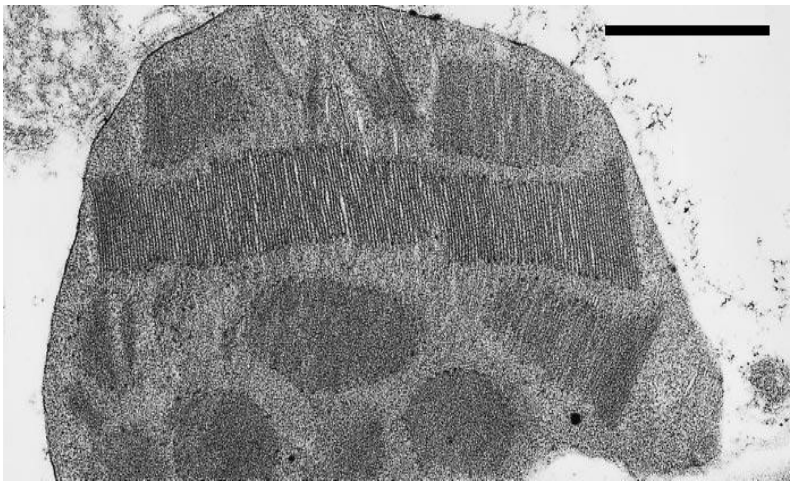


Figure 1: Electron micrographs of a chloroplast from a *Alocasia macrorrhiza* at low irradiance,10 µmole photons $m^{-2}s^{-1}$. (Adapted from Chow *et al.,* 1988)

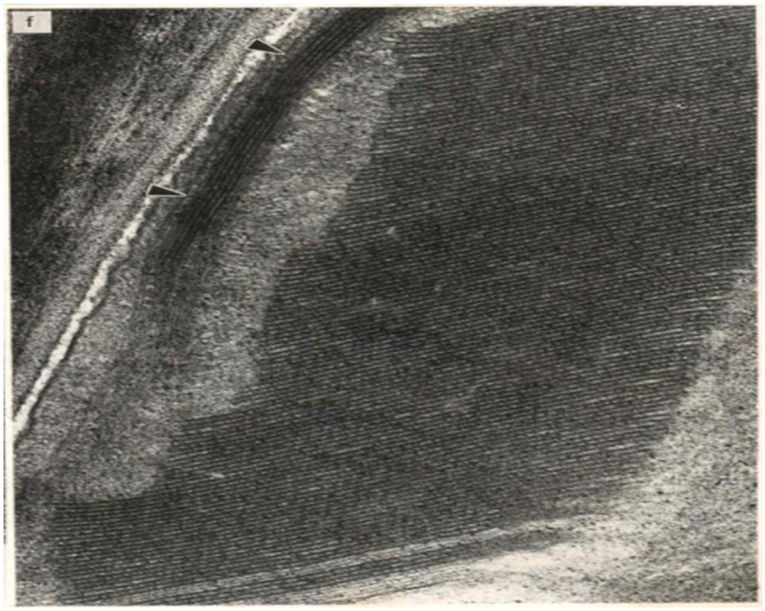


Figure 2 : Large grana each containing over 110 thylakoids in upper epidermal cell plastids of *Teratophyllum rotundifoliatum* (Adapted from from Nasrulhaq-Boyce & Duckett,1991)

More recently, Nasrulhaq-Boyce *et al*. (2011) reported their studies on three species of Malaysian *Pogonatum* collected from habitats exposed to different light intensities which showed the lamellae of the shade *P. cirratum* subsp. *macrophyllum* were rudimentary whilst those in *P. subtortile* and *P. neesii* were between 3-8 cells high (Figure 3). Proctor (2005) reported that lamellae development in ventilated

photosynthetic tissue in some of these plants might enhance the carbon dioxide uptake at high irradiance by providing six or more times to projected area of the leaf.

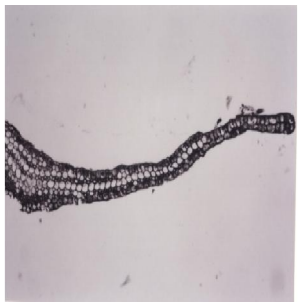
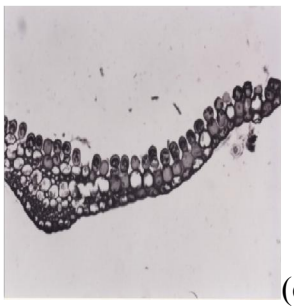
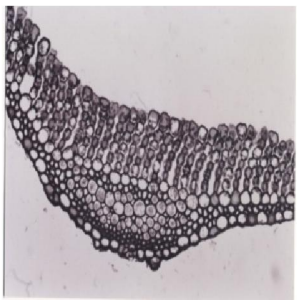
(a)  (b)  (c) 

Figure 3 : Light micrographs of the crosssections of (a) *P. cirratum* subsp. *macrophyllum* leaf, (b) *P. subtortile* leaf showing 2-4 cells high and *Pogonatum neesii* leaf showing 5-7 cells high. (Adapted from Nasrulha-Boyce *et al.,* 2011)

Previous studies on *Guzmania monostachia* leaves grown under high light (650 μmole m^{-2} s^{-1}) have shown that chloroplasts from high-light grown plants had much lower thylakoid content and reduced granal stacking than do in low-light grown plants (Maxwell, 1999). They suggested the acclimation to high light contribute to an adjustment of photosynthetic activity (the level of the content of light-harvesting complex and the amount of Rubisco) and represents an adaptive strategy for the plants to survive in a high light environment.

2.2.5 Photosynthetic activity

The relationship between photosynthesis activity and light intensity of sun and shade plants has been a subject of investigation by plant physiologists for a great many years. Photosynthesis is a conversion of light energy to chemical energy and storing it in the bonds of the carbohydrates, glucose, starch and sucrose. This light-harvesting process takes place in the chloroplast, specifically using the green pigment chlorophyll to capture light from the sun. Early studies by Bohning and Burnside (1956) reported that carbon dioxide uptake by sun plants saturated at a light intensity of 398-498 (μmole m^{-2} s^{-1}) whereas for the shade species it saturated at 99-199 (μmole m^{-2} s^{-1}).

Light saturation rates of carbon dioxide assimilation was higher in most of the sun species (16-20 mg CO_2 dm^{-2} hr^{-1}) compared to the shade species (2-5 mg CO_2 dm^{-2} hr^{-1}). Ludlow and Wolf (1975) reported similar findings from studies with temperate sun and shade ferns. Plants growing under high light intensity showed a higher photosynthetic rate at light saturation than shade plants. However they lower rates at low irradiances (Boardman, 1977; Nasrulhaq-Boyce & Mohamed, 1987). Furthermore, sun plants exhibit greater light compensation points. In addition, sun plants exhibit a higher capacity for photosynthetic electron transport than shade plants (Boardman, 1977; Nasrulhaq-Boyce & Mohamed, 1987). At low light irradiances the rate of electron transport was greater in the sun chloroplasts rather than the shade plant chloroplasts. The lower rates in the shade leaves might be correlated to either the size of the photosynthetic unit (chlorophyll content) or a lower in rate of electron flow through the electron transport chain between the two photosystems.

Boardman (1977) in his review reported that the content of photosynthetic cytochromes *f*, b_{559} and b_{563} were higher in plants that grow in high light irradiances. In support of this, Nasrulhaq-Boyce and Mohamed (1987) reported the amount of protohaem in sun ferns species was higher than shade ferns species. Protohaem is another tetrapyrrole besides chlorophyll which makes up the prosthetic group of cytochromes and is involved in electron transfer activities of chloroplasts and mitochondria. They also reported that chloroplasts from the sun ferns showed greater photochemical rates than did chloroplasts isolated from shade ferns at saturating irradiance. The lower rates of photochemical activity in the shade ferns did not correlate with the difference in amounts of chlorophyll content between sun and shade ferns. However the higher photochemical rates in sun ferns were associated to the greater amounts of electron transport components such as cytochromes *f*, b_{559HP} and b_{559LP} plus b_{563}.

Sun leaves generally have physiological characteristics favouring greater capacity for photosynthesis compared to its shade counterparts. Nasrulhaq-Boyce and Duckett (unpublished data, 1991) reported that the deep shade fern *Teratophyllumn rotundifoliatum*, exhibited a fluctuating diurnal pattern of low photosynthetic rates in its natural habitat. The CO_2 assimilation rates in *T. rotundifoliatum* were saturated (1.4 μmole CO_2 m^{-2} s^{-1}) at very low light irradince.

2.3 Adaptation of Plant to Growth under different light regimes

The relation between light intensity and photosynthesis of individual leaves is important to crop yield in agriculture, horticulture and forestry. Hence, a lot of attention has been given to the effect of light intensity on photosynthetic rates by individual plants. Adaptation to the development of plants at low light apperas to be a question for the economical use of available light. A major factor governing a leaf's photosynthetic productivity is its position in the plant canopy, where it determines its light environment and its rate of net carbon dioxide uptake. The shade plants invest a greater proportion of its synthetic capacity and its maintenance of the light harvesting processes than do sun plants. This is reflected in its chloroplast structure, by enhanced grana development seen in shade plant chloroplasts and the apparent decrease in its stroma relative to chloroplast volume. The light-harvesting in two photosystems (PSI and PSII), together with their reaction centers, increase or at least remain constant in shade plants, while there is a decrease in the level of the soluble proteins, RuDP carboxylase activity and the constituents of the electron transport chain. Plants from low light environments would have use less of these latter compounds. In terms of genetic adaptation to light intensity, studies by Bjorkman (1968) showed that ecological races of the same species from sun and shade habitats marked differently in the adaptation of their photosynthetic response to light intensity. It was previously demonstrated in *Solidago virgaurea*, that clones of

S. virgaurea native to shaded habitats and to exposed environments showed differences in their photosynthetic response to light intensity during their development, where the shaded clones were incapable of adjusting to high intensity, in contrast with clones from sunny habitats, which showed higher light saturated rates of photosynthesis when grown under high light intensities (Bjorkman & Holmgren, 1966). The adaptibility within a genotype could be considerable, although the range of the adjustment varied with different genotypes and it is reflected in the adaptation of genotype to the particular conditions predominate in its natural habitats.

2.4 The filmy ferns

The *Hymenophyllaceae* (filmy ferns) are attractive and can be found most abundant in the humid tropical forest. In *Malay*, it is commonly known as paku *surok-surok*. The *Hymenophyllaceae* ferns is a family of two or more genera, generally restricted to a very damp and shaded environment. Most of the species are found in the tropical rainforest, although some occur in temperate rainforests. They appear as very dark green or even black clumps and sometimes are mistaken for a robust moss or liverworts. A characteristic feature of this family is the very thin lamina of the fronds, where it is usually small in size. The *Hymenophyllaceae* are only found in very moist areas for the greater part of the time, and they can only exist under such conditions. They show a remarkable diversity in terms of morphology and the habitats they occupy. Thus, making the *Hymenophyllaceae* an excellent model group for studying the evolution of ecology and related adaptive survival strategies in Pteridophytes (Dubuisson, 2003).

Generally, the normal structure of leaves consist of an upper and lower epidermis, with several layers of cells in between with inner cells of the leaves being separated from each other by air spaces. Each of the leaves have their own internal air

space for them to communicate with the outer atmospheric air via stomata (tiny pores) in the epidermis layer of cells. In order to survive, plants basically depend on gaseous exchange between the living cells of the leaf and the atmosphere. The epidermial cell function to protect the inner cells while permitting the passage of gasses. However, a different structure is seen in the *Hymenophyllaceae*, where the whole structure is reduced to one single layer of cells. Thus, it tends to have direct immediate contact with the air, and they are able to carry out direct gas-exchange. As the leaves of *Hymenophyllacea*e can absorb water all over their surroundings, and live in wet and damp places, they have a lesser need for conducting water strands seen in most plants and such strands are in fact very small. However, in places that are not constantly wet, most of the *Hymenophyllaceae* must withstand from time to time short periods of drought. Since they have no protection against these conditions, as most other apiphytic ferns, they usually respond merely by wilting and shrivelling themselves. The amount of such drought which they can endure is naturally limited.

Hymenophyllaceae are widely distributed nearly all over the earth, despite their spores being delicate and can not stand the long drought involved in wind-dispersal over long distances. The family is customarily divided into two or more genera. Traditionally, the family of two major clades corresponding to the genera *Hymenophyllum* and *Trichomanes* (Dubuisson *et al.,* 2003). Later, it is divided into 8 genera ; *Abrodictyum, Callistopteris, Cephalomanes, Crepidomanes, Didymoglossum, Polyphlebium, Trichomanes* and *Vandenboschia* (Ebihara *et al*., 2006). Recently, species of *Hymenophyllaceae* contained within *The Plant List* belongs to 31 plant genera. Parris and Latiff (1997) has recorded about 18 species of Hymenophyllaceae in Malaysia. However, this study is only focussing on 3 species, which are *Hymenophyllum, Trichomanes* and *Chephalomanes*.

2.4.1 *Hymenophyllum*

Hymenophyllum is categorized as an epiphyte or rock plant, having a long-creeping rhizome. Their fronds are always arranged in one veined ultimate segments, where the segments could be entire or toothed, glabrous or hairy. The sori of *Hymenophyllum* are at the terminal end of the segments, where it is most commonly on the basal acroscopic segments of the upper pinnae. The lips of their indusium are well developed, and it could be triangular or rounded in shape, entire or toothed. Sometimes it is usually broader and longer than hollow conical basal part with the receptacle usually much shorter than the lips of the indusium. In Malaysia, 14 species of *Hymenophyllum* has been recognized (Holttum , 1968). In contrast with *Trichomanes*, *Hymenophyllum* is characterized by the proportionately longer lips of the indusium, which is the small hollow base of the conical, and also by the complete absence of false veins. Below are some of the *Hymenophyllum* species found in Malaysian highland, area selected for this study.

2.4.1.1 *Hyemenophyllum acanthoides* (Bosch) Rosent

H. acanthoides usually occurs on trees and rocks in mossy mountain forest on the Main Range, although it is also common in the lowlands in the south of Peninsula Malaysia (Figure 4-6). The fronds is strongly crisped, with toothed margins. The indusial lips of its outer surface bears many spines and their margins are also toothed.

Figure 4 : *Hymenophyllum acanthoides*

Figure 5 : Closer look of *Hymenophyllum acanthoides*

Figure 6 : Spines and toothed margins of indusial lips of *H. acanthoides*

2.4.1.2 *Hymenophyllum blandum* Racib.

H. blandum lives on tree trunks and rocks in wet mountain forests. Their fronds are usually small and the lamina is toothed on the margins. *H. blandum* have sori that are protected by an indusium which divides into two rounded lips with toothed edges (Figure 7)

Figure 7: *Hymenophyllum blandum*

2.4.1.3 *Hymenophyllum javanicum* Spreng.

H. javanicum is a common epiphyte found in mountain forest and shady stream banks. The frond lamina is strongly crisped, as that of *H. acanthoides*. However, there are differences in terms of the margin where *H. javanicum* appear to be entire. In dried specimens, the crisping of the lamina is much less obvious. Their toothed indusial lips are longer than the base of the indusium (Figure 8).

Figure 8: *Hymenophyllum javanicum*

2.4.1.4 *Hymenophyllum exsertum* Wall. ex Hook.

This species can be found creeping on the mossy trunk of a young tree on high land mountains. The lamina is flat, with entire margins and the rachis distinctly hairy, making it easier to recognize among the *Hymenophyllum* species (Figure 9 -11).

Figure 9 : The mature *Hymenophyllum exsertum*

Figure 10 : *Hymenophyllum exsertum*

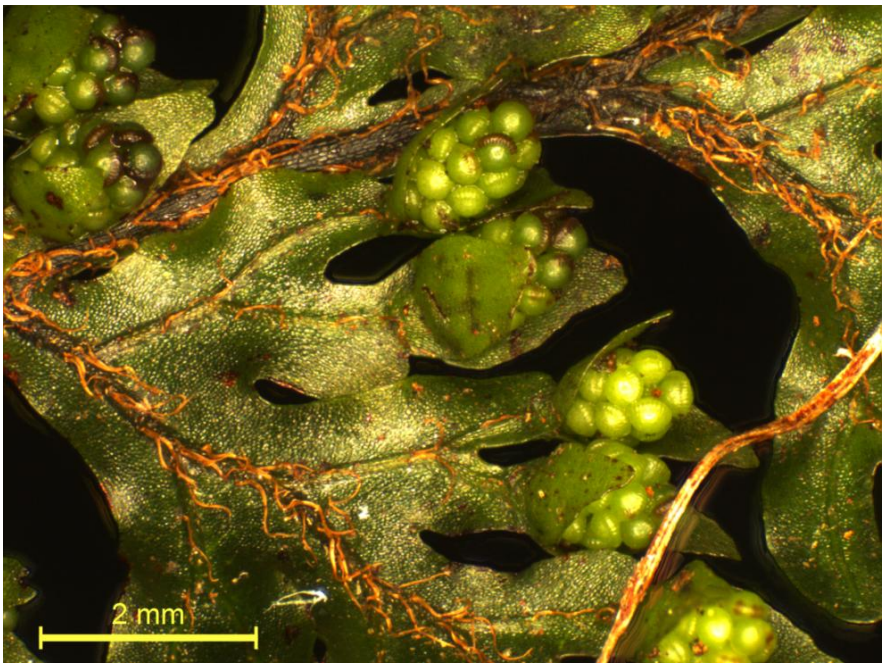


Figure 11: The flat entire lamina and hairy rachis of *H. exsertum*

2.4.1.5 *Hymenophyllum serrulatum* (Presl) C. Chr.

This species is abundant on mossy tree trunks and rocks in mountain forest throughout the Malaysian Peninsula. It can also be found in mangrove areas and freshwater swamp forest in the lowlands in the south. Their fronds are usually long and

the margins of the flat lamina are toothed. The indusial lips are triangular with entire margins (Figure 12).

Figure 12: *Hymenophyllum serrulatum*

2.4.1.6 *Hymenophyllum denticulatum* Sw.

H. denticulatum are locally abundant on mossy tree trunks and rocks in mountain forest and sometimes it can be found in the lowlands in the south of Peninsula. The frond lamina are usually crisped, with toothed margins. The indusium bears a few spines and the margins of the lips are toothed (Figure 13).

Figure 13: *Hymenophyllum denticulatum* (Photo by Ralf Knapp)

2.5.2 *Trichomanes*

*Trichomane*s are different from *Hymenophyllum* in terms of their indusium structure. They have hollow cylindrical or trumpet-shaped indusia with small or no lips, where the hollow portion are very much longer than the lips. Their receptacle usually elongates considerably when mature and projecting well beyond the indusium.

2.5.2.3 *Trichomanes meiofolium* Bory ex Willd.

This species can be found in the mountain, mossy peat forest, on fallen logs and on the base of mossy trees. They have a needle-like leaf structure and the narrow-brist-like segments of the dark green lamina do not lie in a plane but spread in all directions (Figure 14-16).

Figure 14: *Trichomanes meiofolium* on a rotting log at Genting Highlands.

Figure 15: Mature *Trichomanes meiofolium*

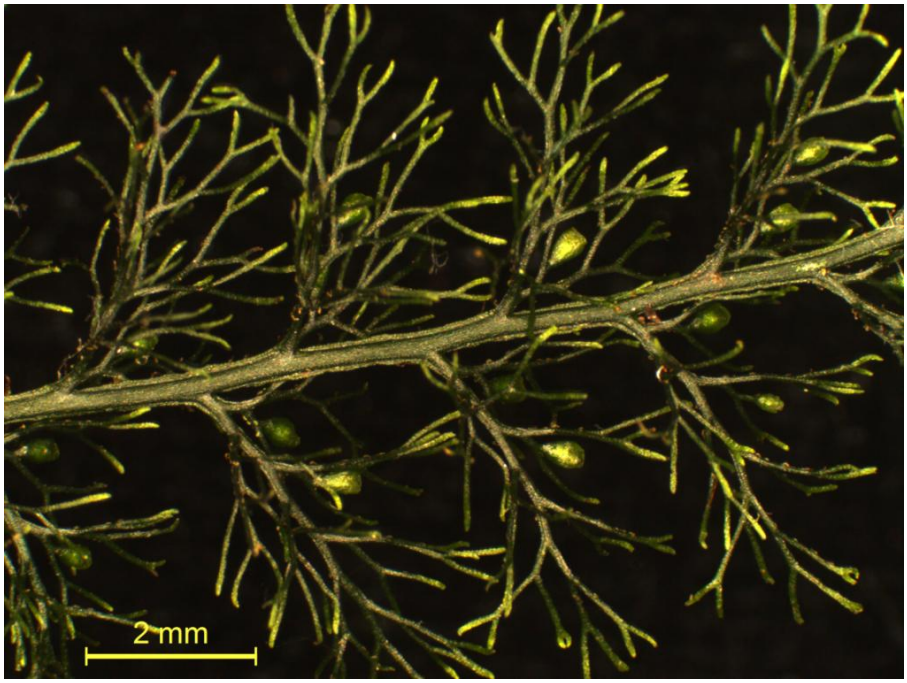


Figure 16 : Needle-like structure of *T. meiofolium*

2.5.3 *Cephalomanes*

2.5.3.1 *Cephalomanes obscurum*

Cephalomanes obscurum is a terrestrial fern, which is erect to 20cm tall. It has 3-pinnate to 3-pinnate-pinnatifid, 5-15 cm long, 2-9 cm wide leaf blade (Figure 20). The cluster of spores (sori) are erect, borne on short lobes in the axils of tertiary segments. This species grows in a damp area, along creek banks or under rock ledges, in a tropical and subtropical rainforest, or in the splash zones of permanent waterfalls (Figure 17).

Figure 17: *Cephalomanes obscurum* found under rock ledges.

2.6 Sun ferns

2.6.1 *Dicranopteris linearis*

This *Gleicheniaceae* species are very common sun ferns in Malaysia, where it can also be called as “Resam” or “Bengkawang” in Malay, and “Mang chi” in Chinese. They survive in the environment with high light irradiances (Figure 18). They form thickets in open places both in the lowlands and in the mountains, and sometimes is a familiar sight on road verges and banks. They also branche with narrower ultimate segments of the lamina. The word “Resam” came from the Arabic verb, which means to delineate. The stem of these ferns can be woven into matting and used to make walls, fish traps, shair seats, caps and pouches. The leaves can be used medicinally as a poltice, and infusions and decoctions in cases of fever or injuries.

Figure 18: *Dicranopteris linearis* densely covering a roadside of the bank in the lowlands.

2.6.2 *Nephrolepis biserrata*

This *Nephrolepidaceae* family are very common terrestrial lowland species, which is adapted in open places, sometimes growing on roacks, and can also be found abundant in plantations, particularly on the trunks of oil palms (Figure 19). These broad sword ferns are sometimes named as ‘Paku uban’ or ‘paku larat’ in Malay. This fern spreads rapidly by means of runners. The rhizome and young fronds are edible and are sometimes used medicinally.

Figure 19: *Nephrolepis biserrata* growing in the lowlands.

Chapter 3

Methodology

3.1 Choice of Plant Materials

The ferns chosen were classified into sun and shade ferns depending on their occurence in a particular light irradiance on the basis that they preferentially grow in their particular habitat, either in sun or in the shade and would not survive in any other habitat. Representative species of the Hymenophyllaceae species mostly growing in the shade were collected from Gunung Ulu Kali, Pahang. The plants studied were collected within altitudinal range between 1300-1700 meters. All selected Hymenophyllaceae species were collected together with the roots and soil. These specimens were placed in plastic bags and brought back to the labroratory for experimental work as soon as possible.

In addition to the samples collected from Gunung Ulu Kali, Pahang, the sun ferns like *Dicranopteri linearis* and *Nephrolepis biserrata* collected from the University of Malaya were also used as experimental material. They were used for experiments on comparing the Rubisco protein expression with the shady Hymenophyllaceae. List for species and specimens collected are presented in **Table 5**.

Table 5. List of species that were collected from Gunung Ulu Kali, Pahang

Species collected	
Shade Species	**Sun Species**
Cephalomanes obscorum	*Dicranopteris linearis*
Hymenophyllum acanthoides	*Nephrolepis biserrata*
H. blandum	
H. denticulatum	
H. exsertum	
H. javanicum	
H. serrulatum	
Trichomanes meiofolium	

3.2 The Site Chosen for Study and Collection

Gunung Ulu Kali is the southernmost mountain in the Main Range in Malaysia and is about 34 miles north-east of Kuala Lumpur. It is situated at the latitude north 3°25.7' and longitude 101°47.5' E, not far from the summit area where the renowned hill resort, Genting Highlands is situated. (**Figure 20**).The plants were collected from the ground and tree trunks in moist and shady forest with surrounding temperature ranging from 19.8°C to 23.8°C. Irradiances and relative humidity measured ranged from 3.3-9.3 µmole m^{-2} s^{-1} and 70-91% respectively.

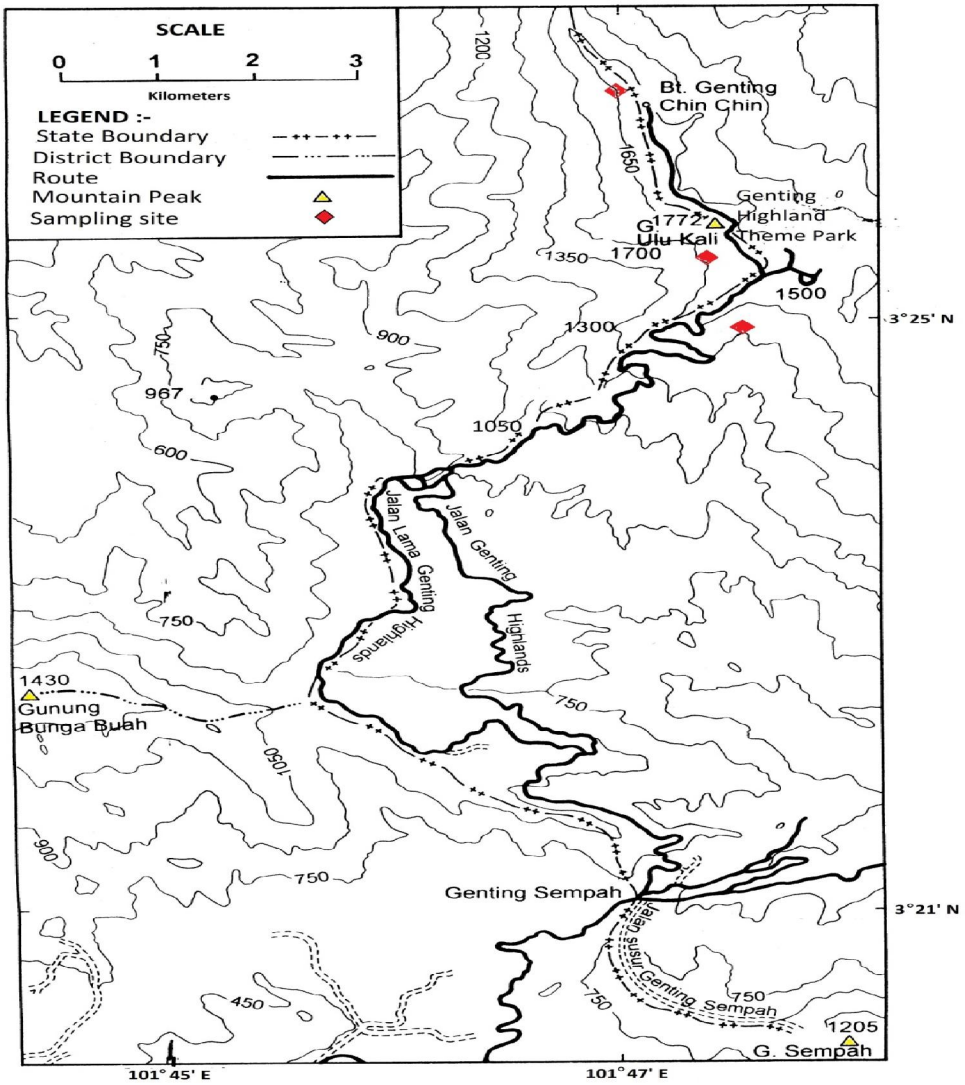


Figure 20. Map of Gunung Ulu Kali, Genting Highlands, Pahang, Malaysia, where the ferns were collected from.

3.3 Chlorophyll determination

In this project the established method of Arnon (1949) was used, as outlined by Nasrulhaq-Boyce and Mohamed (1987). Chlorophyll was extracted by grinding ~0.01g of the leaves in 10ml of 80% acetone in a mortar and pestle. The homogenate was then filtered to remove cellular debris. Absorbance readings of the resulting filtered solution were then taken in a spectrophotometer at 645nm and 663nm, which represents the absorption peaks in 80% acetone of chlorophyll a and b respectively.

The amount of chlorophyll was calculated using the following equation:-

Total chlorophyll = (20.2) (Abs 645) + (8.02) (Abs663) mg/L

Chlorophyll *a* = (12.2) (Abs 663) – (2.69) (Abs 645) mg/L

Chlorophyll *b* = (22.9) (Abs 645) – (4.68) (Abs 663) mg/L

3.4 Protein analysis

Soluble protein was determined following the method of Lowry *et al* (1951). The following reagents were prepared for the purpose:-

A – 2% of Na_2CO_3 anhydrous in 0.1M NaOH

B – 0.5% $CuSO_4$

C – 1.0% sodium potassium tartrate ($NaKC_4H_6O_6.4H_2O$)

D – 48ml of reagent A + 1ml of reagent B + 1 ml of reagent C

(mixed immediately before use)

E – 5ml of Folin-Ciocalteau reagent diluted with 7ml of H_2O

Five replicates of protein suspension with volumes of 0.1ml were first made up to a volume of 1.0ml with distilled water and then mixed with 5.0ml reagent D and allowed to stand for 10 minutes at room temperature. Subsequently 0.5ml of reagent E was added and mixed immediately. After an interval of 30 minutes at room temperature, absorbance measurement was determined at 500nm. The same procedure of standard curve was prepared using known amounts of Bovine Serum Albumin (BSA). A stock solution of 1.0mg/ml was used.

In addition to the protein content of samples analyzed by Lowry protein assay, the Bradford protein assay and Bicinchoninic acid assay was also used in the determination of protein content in the samples. The Bradford assay (Bradford, 1976) is a protein determination assay that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. Under acidic conditions, the dye is predominantly in the red cationic form. However, when the dye binds to protein, it is converted to a stable unprotonated blue form. This blue protein-dye form is detected at 595nm in the assay using a spectrophotometer. Bradford reagent is prepared by dissolving 100mg of Coomassie Brilliant Blue G-250 in 50ml of 95% ethanol, and 100ml 85% (w/v) phosphoric acid. 100 ul of samples extracted is added with 5ml of dye reagent and incubate for 5 min. Then, the absorbance of samples is measured at 595nm. The same procedure of standard curve was prepared using known amounts of Bovine Serum Albumin (BSA). A stock solution of 1.0mg/ml was used.

The Bicinchoninic acid assay (Smith *et al,* 1982) is based on a biuret reaction, which is the reduction of Cu2+ to Cu1+ by protein in an alkaline solution, and a concentration-dependent detection of the monovalent copper ions produced. Bicinchoninic assay is a chromagenic reagent that chelates with the reduced copper,

producing purple reaction complex with strong absorbance at 562nm. In this experiment, BCA protein assay kit from Novagen® was used. After the extraction of fresh leaf samples with phosphate buffer, 50 ul of protein sample replicates is pipetted into labeled test tubes before adding 1.0 ml of BCA working reagent. BCA working reagent is prepared by mixing 1ml of BCA solution with 20 ul of 4% cupric sulfate for each sample. The mixture is vortexes and incubated at 60°C for 15 min. The test tubes are allowed to cool to room temperature. After an interval of 10 min, absorbance measurements were measured at 562nm. The same procedure of standard curve was prepared using known amounts of Bovine Serum Albumin (BSA). The corrected absorbance versus the known mass of the BSA standards is plotted to generate the standard curve. By using the standard curve, the recorded corrected absorbance reading from the samples assayed which fall within the linear range of the standard curve is interpolated. Finally, the amount of protein present in the original sample is calculated.

3.5 Measurement of light intensity, relative humidity and temperature

Light intensity was taken using Luxmeter at each site where the fern species under study occur naturally. Readings were taken between 11.00 am to 2.00 pm in the early afternoon when it was certain that no dark clouds were present in the sky. Readings were taken ten to fifteen times. Same measurements were done for relative humidity and temperature (Spectrum technologies Inc. USA).

3.6 Chlorophyll fluorescent measurements

Chlorophyll fluorescent provide detailed information on the saturation characteristics of electron transport, as well as the overall photosynthetic performance of a plant (Ralph & Gademann, 2005). They proposed that low light leaves showed

limited photosynthetic capacity and reduced activity of non-photochemical quenching pathways compared with high light leaves, where their photosynthesis were not limited and it showed an elevated level of non-photochemical quenching. Generally, chlorophyll fluorescence is a light energy absorbed by chlorophyll molecules in a leaf and it can be re-emitted as light, instead of photosynthesis purposes and dissipated as heat. Thus, by measuring the yield of chlorophyll fluorescence, information about changes in the efficiency of photochemistry and heat dissipation can be obtained (Maxwell & Johnson, 2000).

The photochemical quenching parameters always relate to the relative values of Fm and Fv, where the most useful parameters that measure the efficiency of Photosystem II photochemistry, PSII. This parameter will measure the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry. The photochemical quenching can also be interrelated by Fv/Fm parameter, where it measures the maximum efficiency of PSII. A change in Fv/Fm is because of the non-photochemical quenching in PSII. Thus, this dark-adapted values of Fv/Fm are used as a sensitive indicator of plant photosynthetic performance, with optimal values of around 0.83 measures for most plant species (Bjorkman & Demmig, 1987). Values which is lower than 0.83 will be seen when the plant is under stress (which indicates the phenomenon of photoinhibition). The chlorophyll fluorescence measurements were made using a modulated chlorophyll fluorometer. The sample material was placed in standard Hansatech leaf clips and the leaves were measured in dark adapted for 10 min.

3.7 Gas exchange and photosynthetic rate measurements

Gas exchange (*In vivo* photosynthetic rates) measurements were made with a portable Infra-red gas analyzer LICOR photosynthesis system. The Infra-Red Gas

Analyzer is a reliable and convenient instrument to determine photosynthetic CO_2 fixation and photorespiratory CO_2 release in plants. Measurements of light response curve were taken with a range of PAR 0-2000 μmol m^{-2} s^{-1}. Light intensity (Photosynthetically active radiation, PAR) within the sampling chamber was set at 2000 μmol $m^{-2}s^{-1}$, using a Li-6400-02B LED light source (LI-COR). The CO_2 flow into the chamber was maintained at a concentration of 400 μmol mol^{-1} using a LI-6400-01 CO_2 mixer (LI-COR). The humidity flow into the chamber was fixed at 500 μmol s^{-1}, and desiccant mid-range between scrub and bypass. All measuremnets was done on gas exchange parameters between 0800 to 1200 h, which was presumed photosynthetic rates would be maximal (DiCristina & Germino, 2006). Same procedure was followed for photosynthetic rate, where the parameters were taken in the early afternoon when it was certain that no dark clouds were present in the sky.

3.8 Microscopy

Fresh leaves were cut into small pieces and then mounted on a slide using distilled water as medium. The slide was examined and photographed using compound microscope model Leica DM1000. Chloroplast size and its number per cell were calculated from 20 different cells, with four cells each of five different leaves.

3.9 Plant protein extraction

A fresh leaf tissue is transferred to a cooled mortar and grind in liquid nitrogen until became white powder. A 50 to 150 mg of sample tissues were diluted to 1ml of extraction buffer (100mM of Tris-HCl, pH 8.3, 5mM EDTA, 100mM of KCl, 1% of DTT, 30% of sucrose and protease inhibitor). The 1 ml of cell lysate was mixed with 8 ml of 100% ice-cold acetone and 1 ml of 100% trichlorocetic acid (TCA) according to

1:8:1 ratio. The mixture solution was vortexed and precipitated at -20 C for 1 hour before centrifuge at 11,500 rpm (18,000 x g) for 15 min at 4 C in a microcentrifuge. The supernatant was discarded and 1ml of 100% ice cold acetone was added to wash the pellet. The sample was incubated on ice for 15min and centrifuged as above. The acetone-containing supernatant was removed and the pellet was removed and the pellet was air dried. For 1D gel electrophoresis, the pellet was suspended in 200 µl of sample buffer, consisting of 7M urea, 2M thiourea, 20mM Tris, pH 7.5, 2% 3-[(cholamidopropyl) dimethylamino]-1-propanesulfonate (CHAPS), 0.4%-dithiothreitol by repeatedly pipetting up and down to break up the pellet. The sample is incubated at room temperature for 1 hour and vortexing approximately every 10 min before centrifuge at 14,000 rpm for 10 min at 4 C. The supernatant is transfered into a new eppendorf tube and stored at -80 C until ready for use. The total protein was determined by the method utilized by Bradford (1976), against a standard curve prepared with bovine serum albumin.

3.10 SDS-PAGE and Rubisco expression detection

According to Boardman (1977), the higher maximum photosynthetic rate in plants grown in high light is due to higher concentrations of photosynthetic enzymes like ribulose bisphosphate carboxylase/oxygenase (Rubisco) in leaf tissue. Rubisco generally accounts for more than 50% of the protein leaf tissue. The higher amount of Rubisco in leaves is important since it is the first enzyme involved in acquiring carbon from the atmosphere and converting the carbon into sucrose and other carbohydrates that can be used to support plant growth. In this experiment, we determined the amount of Rubisco expressed in shade and sun ferns using SDS-PAGE gel electrophoresis and

Western Blot. Western Blot is a procedure that allows the isolation and detection of a particular protein using antibodies designed to specifically bind to that protein.

Analytical polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed with 12% acrylamide gel according to the method of Laemmli (1970). Samples were prepared for electrophoresis by boiling for 3 min in the presence of 2% (w/v) SDS and 0.1M B-mercaptoethanol. Protein bands were stained with silver nitrate according to Heukeshoven and Dernick (1986). Molecular mass of protein bands were estimated by using the marker proteins (Bio-Rad & GeneDirex).

For Western Blot, proteins were denatured and separated by size in 12% SDS-PAGE. The gel is a matrix through which the denatured proteins will migrate based on their molecular weight. Small proteins will move further along in the gel and large proteins will move shorter distances. Proteins are then transferred to Pall, Biotrace nitrocellulose transfer membrane (medigene) and probing with rabbit anti-Rubisco polyclonal antibody, HRP conjugated. The reaction with antibodies was carried out according to Fereira and Janick (1996). The membrane is then incubated in a substrate that reacts with the reporter enzyme to produce stained bands. The density of the stained band can be used to estimate the relative amounts of the specific protein from different protein samples.

3.11 Statistic analysis

The data obtained were pooled and analysed using Minitab Pro v16.1.0.0 statistical software. A one-way ANOVA was applied to evaluate significant differences in the studied parameters. The least significant difference was calculated following a significance at p=0.05. The Tukey's Honest Significance Difference (HSD) Test, at α =

0.05 level of significance was also done to compare the means and to determine whether there were any differences among the morphological and physiological parameters between treatments. All parameters obtained were expressed as mean $\pm$ S.E. from measurements.

CHAPTER 4

RESULTS

4.1 Environmental measurements of the habitats from which the species were collected

The Hymenophyllaceae species collected in the present study were generally from constantly moist, shaded habitats. **Table 6** shows that the irradiances were considerably low for the eight species of Hymenophyllaceae where the light intensities received by the plants were below ~100 μmole m^{-2} s^{-1}. The light intensity increased in the morning as a result of sunrise but remained low and fairly constant until early afternoon after which it started to decrease. Most of the Hymenophyllaceae species studied were found in shaded habitats and under moist conditions. The temperature and relative humidity of the habitats of the Hymenophyllaceae species ranged from 19.8- 23.8 °C and 69.9- 91.4%, respectively.

Light intensity measurements were taken between 11.00 to 12.00pm where each species naturally grow, using a light meter. Readings were taken on days in which the sky was not overcast and there was maximum sunlight.

Table 6. A summary of environmental measurements in the habitats from which the species were collected.

Species	Irradiances (µmole $m^{-2}s^{-1}$)	Temperature (°C)	Relative humidity (%)
Cephalomanes obscurum	33.3 ± 0.35	21.90 ± 0.54	89.20 ± 0.25
Hymenophyllum acanthoides	92.2 ± 1.41	22.60 ± 0.49	84.90 ± 1.30
H. blandum	60.0 ± 0.38	21.20 ± 0.72	73.10 ± 0.82
H. denticulatum	69.9 ± 0.49	22.50 ± 0.22	90.80 ± 0.28
H. exsertum	58.7 ± 0.29	21.10 ± 0.72	69.90 ± 2.30
H. javanicum	93.3 ± 0.81	23.80 ± 0.67	82.80 ± 0.35
H. serrulatum	60.1 ± 0.69	21.70 ± 0.72	82.60 ± 2.91
Trichomanes meiofolium	45.4 ± 0.58	19.80 ± 0.31	91.40 ± 0.28

All values are expressed as mean ± SE.

4.2 Leaf Chlorophyll Content of Hymenophyllaceae species and two sun ferns

Chlorophyll content expressed on a fresh weight basis was exceptionally high in *T. meiofolium* (8.6 mg g^{-1}), followed by the other species ranging between 3.3-6.8 mg g^{-1} fresh weight (**Table 7**). *O. obscorum, H. serrulatum* and *H. denticulatum* showed chlorophyll content values of 6.8 mg g^{-1} fresh weight , 6.3 mg g^{-1} fresh weight and 5.8 mg g^{-1} fresh weight, respectively. Whilst, the other species expressed chlorophyll values of 4.7 mg g^{-1} fresh weight (*H. javanicum),*3.8 mg g^{-1} fresh weight *(H. acanthoides),* 3.5 mg g^{-1} fresh weight *(H. exsertum),* and 3.3 mg g^{-1} fresh weight *(H. blandum).* For comparison purposes, the two sun ferns were collected, *Dicranopteris linearis* and *Nephrolepis biserrata,* exhibited much lower amount of chlorophyll, where their values ranged between 1.6 to 2.6 mg g^{-1} fresh weight. All the *Hymenophyllaceae* species recorded higher chlorophyll *a* content than the two sun ferns studied. However, in terms of chlorophyll *b* content, *H. acanthoides, H. blandum, H. exsertum* and *H. javanicum* exhibited content similar to the sun ferns.

With regard to chlorophyll *a/b* ratio, the *Hymenophyllaceae* species exhibited a lower ratio, ranging between 0.9~1.9, whilst, the sun ferns (*Dicranopteris linearis* and *Nephrolepis biserrata*) showed a higher chlorophyll *a/b.*

Table 7. Leaf chlorophyll content of Hymenophyllaceae species and two sun ferns.

Species	Total chlorophyll (mg g^{-1} fresh weight)	Chlorophyll a (mg g^{-1} fresh weight)	Chlorophyll *b* (mg g^{-1} fresh weight)	Chlorophyll *a/b* ratio
Shade				
Cephalomanes obscorum	$6.80^{ab} \pm 0.40$	$3.30^{bcd} \pm 0.20$	$3.60^{a} \pm 0.20$*	$0.90^{d} \pm 0.04$*
Hymenophyllum acanthoides	$3.80^{bcd} \pm 0.8$	$2.50^{bcde} \pm 0.50$	$1.30^{cde} \pm 0.30$	$1.90^{bc} \pm 0.01$
H. blandum	$3.30^{cd} \pm 0.50$	$2.10^{cde} \pm 0.30$	$1.40^{cde} \pm 0.30$	$1.70^{c} \pm 0.13$*
H. denticulatum	$5.80^{abc} \pm 0.20$	$3.80^{abc} \pm 0.10$	$2.00^{c} \pm 0.10$*	$1.90^{b} \pm 0.01$*
H. exsertum	$3.50^{cd} \pm 0.40$	$2.20^{bcde} \pm 0.30$	$1.30^{cde} \pm 0.10$	$1.80^{bc} \pm 0.09$
H. javanicum	$4.70^{bcd} \pm 0.50$	$2.90^{bcde} \pm 0.30$	$1.70^{cd} \pm 0.20$	$1.70^{bc} \pm 0.04$
H. serrulatum	$6.30^{abc} \pm 1.60$	$4.10^{ab} \pm 1.10$	$2.20^{bc} \pm 0.60$	$1.90^{bc} \pm 0.03$
Trichomanes meiofolium	$8.60^{a} \pm 0.30$*	$5.60^{a} \pm 0.20$*	$3.10^{ab} \pm 0.10$	$1.80^{bc} \pm 0.02$
Sun				
Dicranopteris linearis	$2.60^{d} \pm 0.20$	$1.80^{de} \pm 0.10$	$1.80^{de} \pm 0.10$	$2.30^{a} \pm 0.02$
Nephrolepis biserrata	$1.60^{d} \pm 0.20$	$1.20^{e} \pm 0.10$*	$1.20^{e} \pm 0.10$*	$2.60^{a} \pm 0.05$

All values are expressed as mean ± SE. Means that do not share a letter are significantly different at alpha = 0.05
*Indicates significance at 5% level.

4.3 Leaf Soluble Protein Content of Hymenophyllaceae species and two sun ferns determined via the Lowry, Bradford and BCA methods

Contrary to the level of chlorophyll, the soluble protein content were generally lower in all Hymenophyllaceae species, except in *H. serrulatum,* compared with the two sun-type ferns, *Dicranopteris linearis* and *Nephrolepis biserrata.* Soluble protein content in *H. serrulatum* was remarkably high when all the three methods used, 53 mg/g fresh weight for Lowry, 34 mg/g fresh weight for BCA and 17.0 mg/g fresh weight for Bradford. However the protein content determined by the Bradford assay, was lower when compared with *Nephrolepis linearis*. Using the Lowry protein assay, *H. denticulatum, H. acanthoides, T. meiofolium, C. obscorum*, *H. javanicum, H. exsertum* and *H. blandum* exhibited protein content ranging between 0.3 to 9.7 mg/g fresh weight (**Table 8**). Whilst, for the Bradford protein assay, *H. serrulatum, T. meiofolium, H. acanthoides., C. obscorum, H. denticulatum, H. javanicum, H. exsertum* and *H. blandum* exhibited protein values ranging between 1.6 to 17 mg/g fresh weight. The soluble protein content exhibited in *H. javanicum, H. acanthoides, H. exsertum, H. blandum, T. meiofolium, C. obscorum* and *H. denticulatum* ranged between 0.3 to 2.4 mg/g fresh weight, when the BCA protein assay was used. With regard to protein/chlorophyll ratio, *Dicranopteris linearis* and *Nephrolepis biserrata* expressed higher values compared to all the Hymenophyllaceae studied.

Table 8. Leaf soluble protein content of Hymenophyllaceae species and two sun ferns determined via the Lowry, Bradford and BCA methods.

Species	Soluble protein content					
	Lowry		Bradford		BCA	
	Protein content (mg g^{-1})	Protein/chl ratio	Protein content (mg g^{-1})	Protein/chl ratio	Protein content (mg g^{-1})	Protein/chl ratio
Shade						
Cephalomanes obscurum	$1.3^{d} \pm 0.02$	$0.2^{d} \pm 0.01$	$5.1^{d} \pm 0.11$	$0.8^{d} \pm 0.04$	$3.0^{ef} \pm 0.23$	$0.4^{bc} \pm 0.02$
Hymenophyllum acanthoides	$8.6^{bc} \pm 0.70$	$2.9^{cd} \pm 0.79$*	$5.5^{d} \pm 0.12$	$1.8^{cd} \pm 0.46$*	$8.4^{d} \pm 1.04$*	$2.9^{b} \pm 0.85$*
H. blandum	$0.3^{d} \pm 0.03$	$0.1^{d} \pm 0.02$	$1.6^{e} \pm 0.12$	$0.5^{d} \pm 0.06$	$4.1^{e} \pm 0.10$	$1.4^{bc} \pm 0.21$
H. denticulatum	$9.7^{bc} \pm 0.70$	$1.7^{d} \pm 0.12$	$5.1^{d} \pm 0.19$	$0.9^{d} \pm 0.04$	$1.5^{f} \pm 0.02$*	$0.3^{c} \pm 0.01$*
H. exsertum	$0.4^{d} \pm 0.05$	$0.1^{d} \pm 0.01$	$1.8^{e} \pm 0.09$	$0.5^{d} \pm 0.03$	$4.6^{e} \pm 0.33$	$1.3^{bc} \pm 0.08$
H. javanicum	$1.2^{d} \pm 0.06$	$0.3^{d} \pm 0.03$	$2.9^{e} \pm 0.15$	$0.6^{d} \pm 0.09$	$10^{cd} \pm 0.82$	$2.4^{bc} \pm 0.39$
H. serrulatum	$53^{a} \pm 3.50$*	$10^{a} \pm 0.02$*	$17^{b} \pm 0.49$*	$3.3^{bc} \pm 0.60$*	$34^{a} \pm 0.83$*	$5.9^{a} \pm 0.95$
Trichomanes meiofolium	$4.6^{cd} \pm 0.20$	$0.5^{d} \pm 0.03$	$5.6^{d} \pm 0.05$	$0.7^{d} \pm 0.03$	$4.0^{ef} \pm 0.12$	$0.5^{bc} \pm 0.03$
Sun						
Dicranopteris linearis	$14^{b} \pm 0.28$	$5.5^{bc} \pm 0.47$*	$13^{c} \pm 0.37$*	$5.4^{b} \pm 0.53$*	$18^{b} \pm 0.51$*	$7.3^{a} \pm 0.82$
Nephrolepis biserrata	$12^{b} \pm 0.25$	$7.6^{ab} \pm 0.50$*	$20^{a} \pm 0.67$*	$12^{a} \pm 1.04$*	$12^{c} \pm 0.22$*	$7.8^{a} \pm 0.58$

All values are expressed as mean ± SE. Means that do not share a letter are significantly different at alpha = 0.05
*Indicates significance at 5% level.

4.4 Mesophyll chloroplast number in the leaves of the Hymenopyllaceae species

It has been well documented that shaded ferns have larger and fewer chloroplasts but are richer in chlorophyll content when compared to sun ferns (Boardman, 1977; Nasrulhaq-Boyce & Mohamed, 1987; Nasrulhaq-Boyce & Duckett, 1991). Data for chloroplast number and size are presented in **Table 9**. Light microscopy observations showed that *C. obscorum* had the highest number of chloroplast per cell profile, numbering 138, followed by *H. acanthoides (63), H. serrulatum (54), H. exsertum (46), H. javanicum (43), H. denticulatum (42),* and *H. blandum (34)*. However with regards to size, the chloroplast were significantly larger in *H. blandum* (6.5 µm) , than in C. obscurum, *H. acanthoides, H. serrulatum*, *H. exsertum, H. denticulatum* and *H. javanicum.* Chloroplast size for *H. denticulatum, H. serrulatum, H. exsertum, H. javanicum, H. acanthoides* and *C. obscorum* were 6.4µm, 6.1µm, 5.7µm, 5.7µm, 5.6µm and 4.8µm, respectively. *C. obscorum*, exhibited the highest number of chloroplasts and the smallest chloroplast size, It showed an extraordinary cell wall shape which was very different from the other species. Nevertheless the chloroplast numbers recorded are relatively low and under the light microscope the cells can be seen to have closely packed chloroplasts (**Figure 21-27**).

Table 9. Mesophyll chloroplast number per profile in the leaves of the Hymenophyllaceae species.

Species	Chloroplast number per cell profile	Chloroplast size (µm)
Cephalomanes obscurum	$138^{a} \pm 3$*	$4.80^{c} \pm 0.08$*
Hymenophyllum acanthoides	$63^{b} \pm 3$*	$5.60^{b} \pm 0.14$
H. blandum	$34^{e} \pm 1$*	$6.50^{a} \pm 0.15$
H. denticulatum	$42^{de} \pm 1$	$6.40^{a} \pm 0.11$
H. exsertum	$46^{cd} \pm 2$	$5.70^{b} \pm 0.11$
H. javanicum	$43^{de} \pm 2$	$5.70^{b} \pm 0.09$
H. serrulatum	$54^{bc} \pm 2$	$6.10^{bc} \pm 0.16$

All values are expressed as mean ± SE. Means that do not share a letter are significantly different at alpha = 0.05
*Indicates significance at 5% level

Figure 21 : Light micrographs showing chloroplasts in leaf cells of *H. serrulatum*, x 40

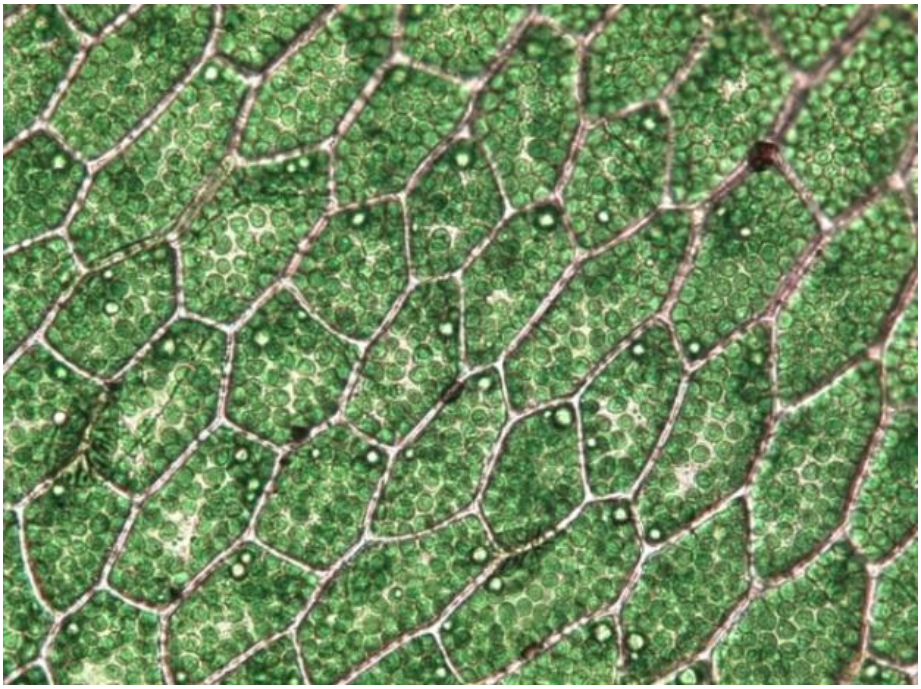


Figure 22: Light micrographs showing chloroplasts in leaf cells of *H. javanicum*, x 40

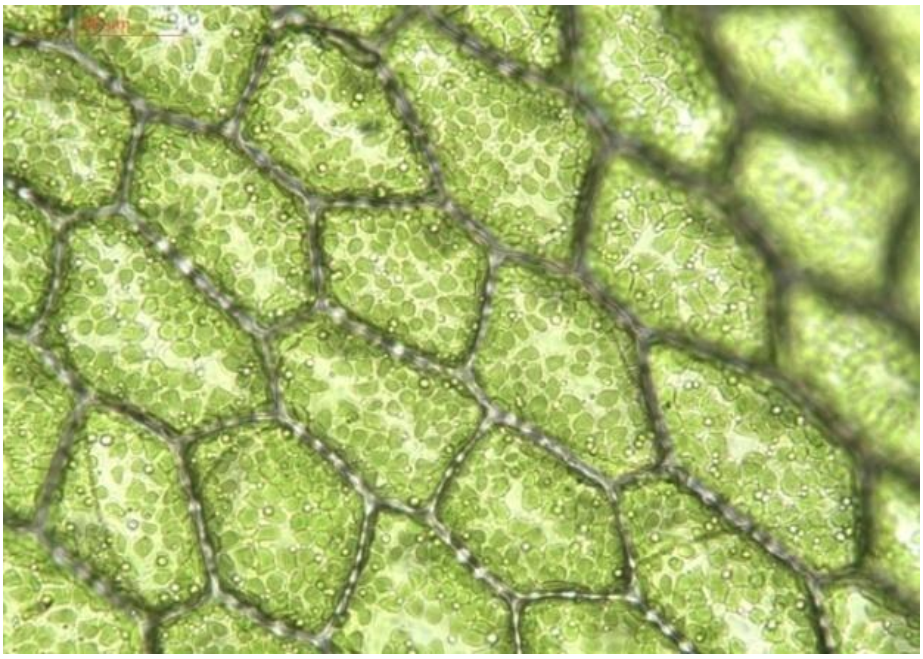


Figure 23: Light micrographs showing chloroplasts in leaf cells of *H. acanthoides,* x 40

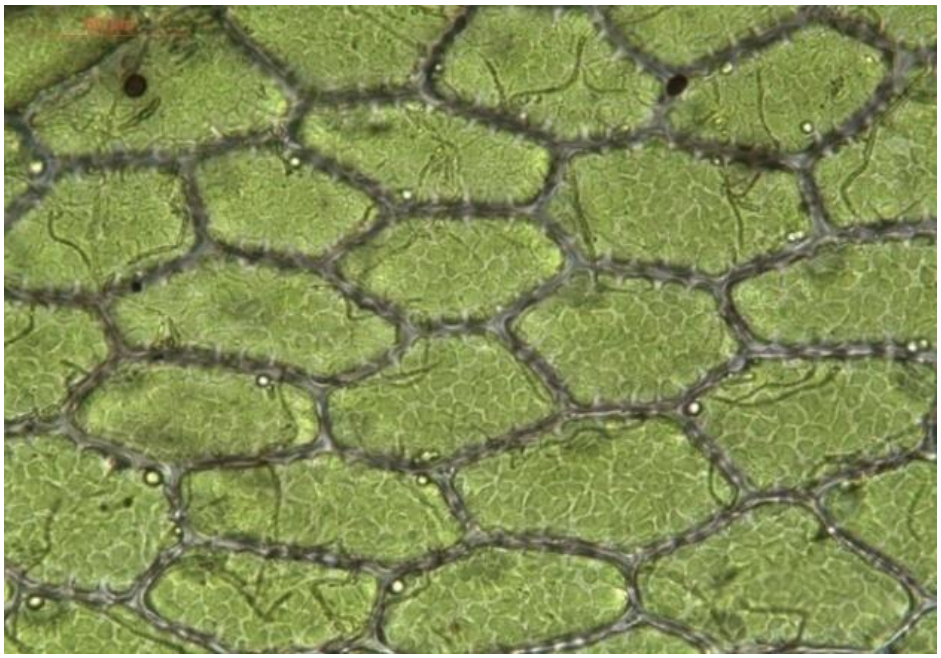


Figure 24: Light micrographs showing chloroplasts in leaf cells of *H. exsertum*, x 40

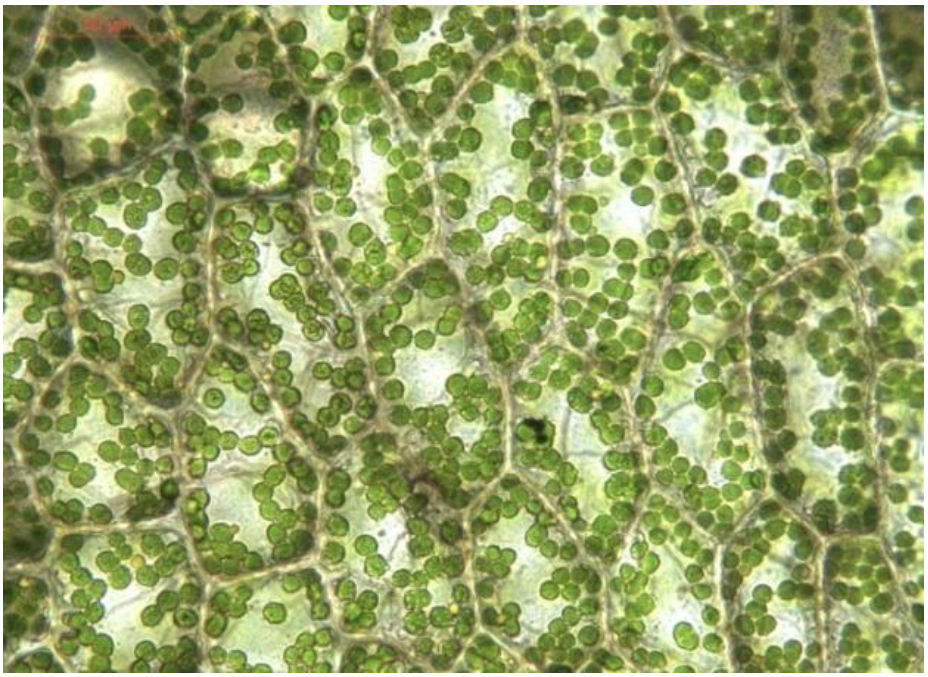


Figure 25: Light micrographs showing chloroplasts in leaf cells of *H. blandum*, x 40

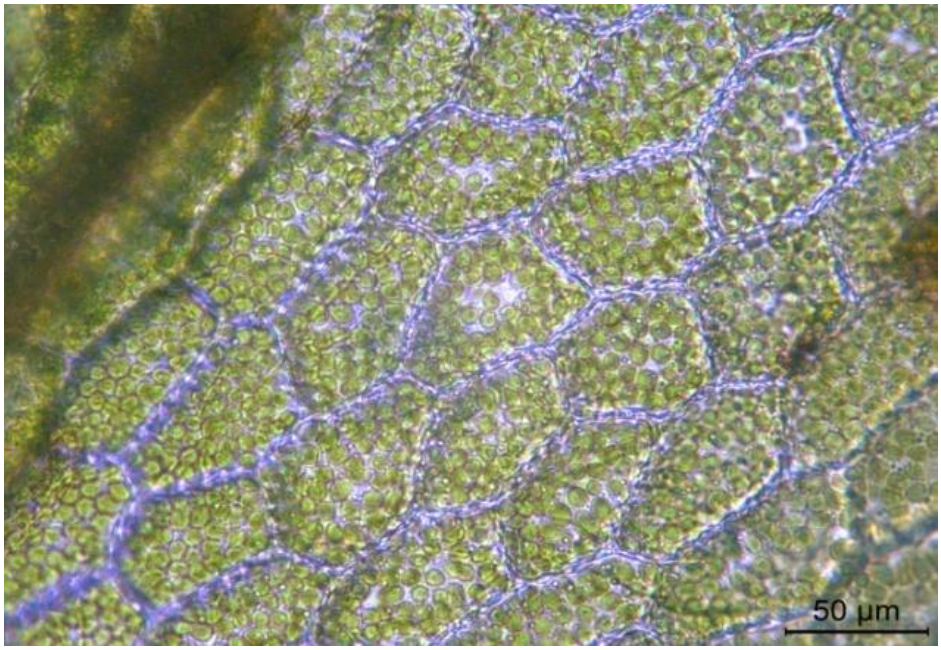


Figure 26: Light micrographs showing chloroplasts in leaf cells of *Hymenophylllum denticulatum*, x 40

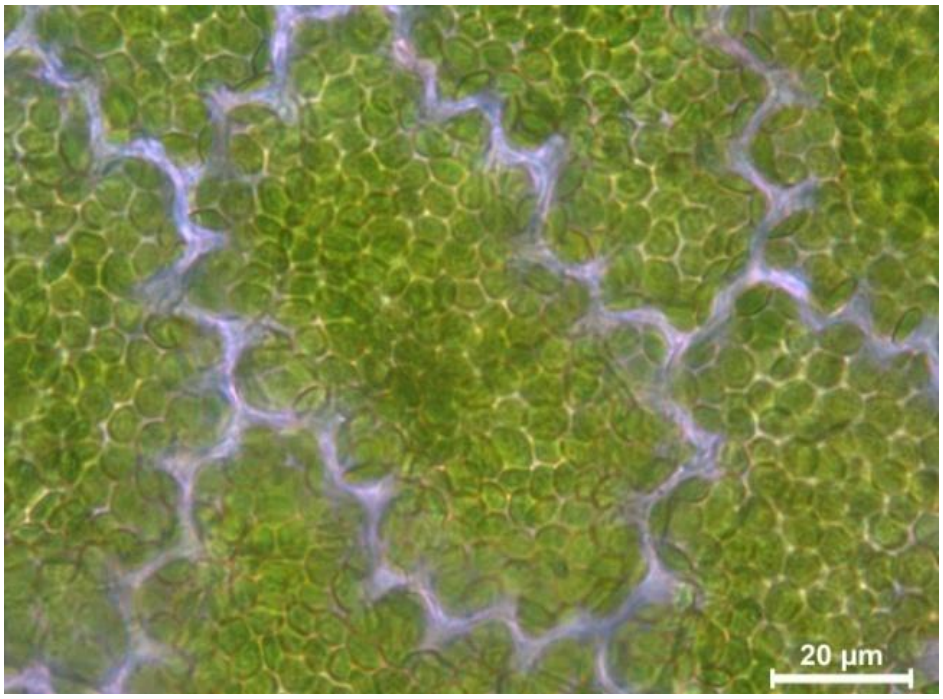


Figure 27: Light micrographs showing chloroplasts in leaf cells of *Cephalomanes obscurum*, x 120

4.5 Chlorophyll Fluorescence and Photosynthetic Light Response Curves in the leaves of the Hymenophyllaceae species collected

The efficiency of photochemical quenching can be determined by comparing Fm (maximal fluorescence) values and the yield of fluorescence in the absence of photosynthetic light, Fo (minimal fluorescence). Fv (variable fluorescence) is calculated as Fv = Fm-Fo. This ratio of Fv/Fm (variable fluorescence to maximal flourescence) can be used to estimate the potential efficiency of PSII by taking the dark-adapted measurements. Chlorophyll fluorescence in *H. denticulatum, H. javanicum, T. meiofolium* and *H. serrulatum* exhibited Fv/Fm ratios or photosynthetic quantum yield values, ranging between 0.71 to 0.81 (**Table 10**) which are values within the range for a normal healthy leaf. Lower Fv/Fm values were seen in three species (*H. serrulatum, H. javanicum* and *T. meiofolium*), where the values recorded were 0.73, 0.74 and 0.77, respectively. *H. denticulatum* recorded the highest Fv/Fm value (0.81). The variable fluorescence (Fv) and the maximal fluorescence (Fm) of *H. denticulatum* were the lowest values recorded, 379 and 1955, respectively. As shown in Table 10, *H. serrulatum, H. javanicum* and *T. meiofolium* were probably under stress (due to photoinhibition) because their Fv/Fm values (< 0.8) were low.

Figures 28 to 37 show the *in vivo* light saturation curve for photosynthesis in the *Hymenophyllaceae* species and in some selected sun ferns, which were determined using a portable Infra-red Gas Analyzer Photosynthesis System. Initial *in vivo* light saturation studies on eight species, *H. serrulatum*, *T. meiofolium*, *H. exsertum, H. denticulatum, H.*

javanicum, H. acanthoides, H. blandum and *Chephalomanes obscurum* showed that CO_2 assimilatory rates for all ferns were low ranging between 3 to 15 µmol CO_2 m^{-2} s^{-1}. The shade-adapted *H. blandum* recorded the lowest photo-assimilatory rates (~3 µmol CO_2 m^{-2} s^{-1}) compared to *H. jvanicum* (~5 µmol CO_2 m^{-2} s^{-1}), *H. serrulatum* (~9 µmol CO_2 m^{-2} s^{-1}), *Hymenophyllum denticulatum* (~10 µmol CO_2 m^{-2} s^{-1}), and *Trichomanes meiofolium* (~11 µmol CO_2 m^{-2} s^{-1}). Meanwhile, *H. exsertum, H. acanthoides* and *Chepalomanes obscorum* share the highest photosynthetic rates, approximately 14 to 17 µmol CO_2 m^{-2} s-1. Even though photosynthetic activity fluctuated over a period of time it was definitely positive throughout the duration of experiments. The photosynthetic rates fluctuated throughout the day independently of light intensity that came through to the forest floor.

These plants showed optimal photosynthesis at light intensities between 100 to 150 µmole $m^{-2}s^{-1}$, a light intensity to which they are normally exposed to in their native environment, except for *H. blandum* which saturated at around 300-400 µmole $m^{-2}s^{-1}$. In contrast, the sun ferns *Dicranopteris linearis* and *Nephrolepis biserrata*, showed higher CO_2 saturation rates, 22 µmol CO_2 m^{-2} s^{-1} and 30 µmol CO_2 m^{-2} s^{-1}, respectively. The sun ferns showed optimal photosynthesis at higher light irradiance (~600µmole $m^{-2}s^{-1}$) than what was observed in shaded species.

Table 10. Chlorophyll fluorescence in the leaves of the Hymenophyllaceae species collected.

Species	Fv	Fm	Fv/Fm
Hymenophyllum denticulatum	$379^{b} \pm 39$*	$1955^{a} \pm 145$	$0.810^{a} \pm 0.009$*
H. javanicum	$569^{a} \pm 42$*	$2205^{a} \pm 75$	$0.740^{b} \pm 0.010$
H. serrulatum	$512^{ab} \pm 49$	$1963^{a} \pm 196$	$0.730^{b} \pm 0.020$
Trichomanes meiofolium	$510^{ab} \pm 26$	$2228^{a} \pm 112$	$0.770^{ab} \pm 0.010$

All values are expressed as mean ± SE. Means that do not share a letter are significantly different at alpha = 0.05
*Indicates significance at 5% level

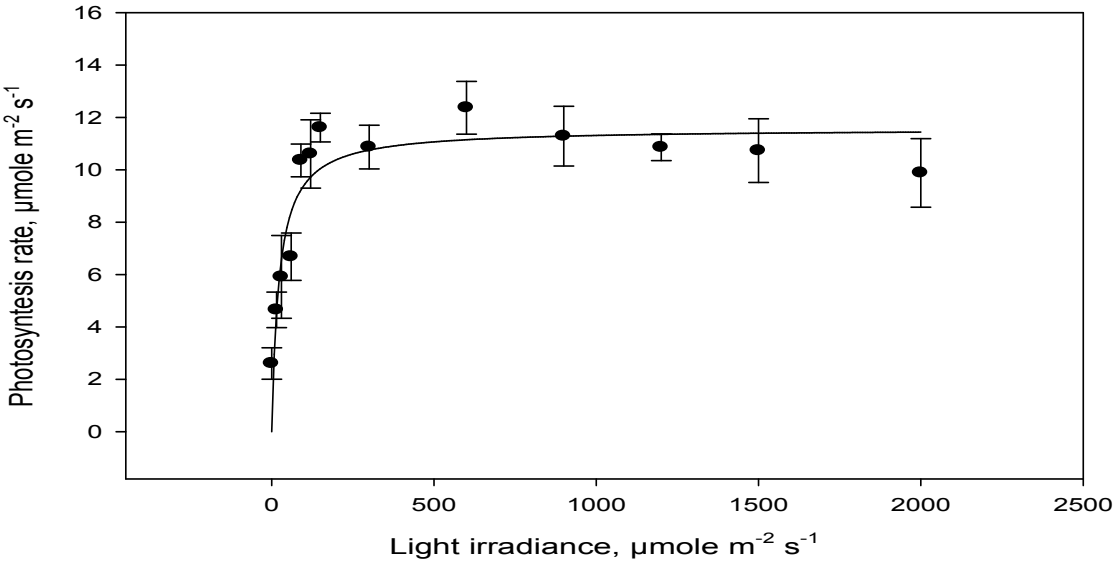


Figure 28: The effect of varying light intensity on the photosynthetic rates in the leaves of *Trichomanes meiofoloium.* Points show the mean values calculated from 3 readings.

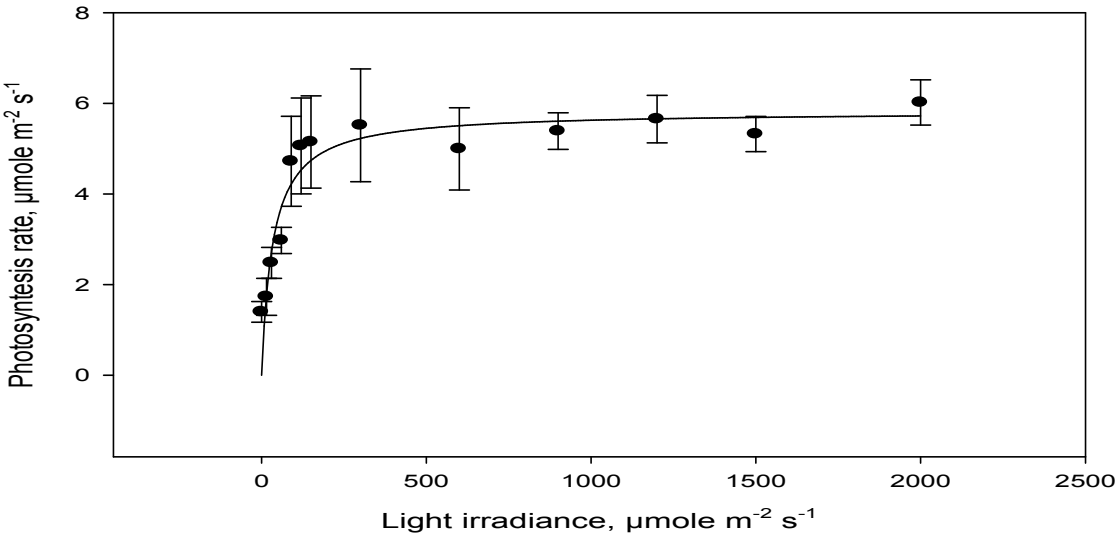


Figure 29: The effect of varying light intensity on the photosynthetic rates in the leaves of *Hymenophyllum javanicum.* Points show the mean values calculated from 3 readings.

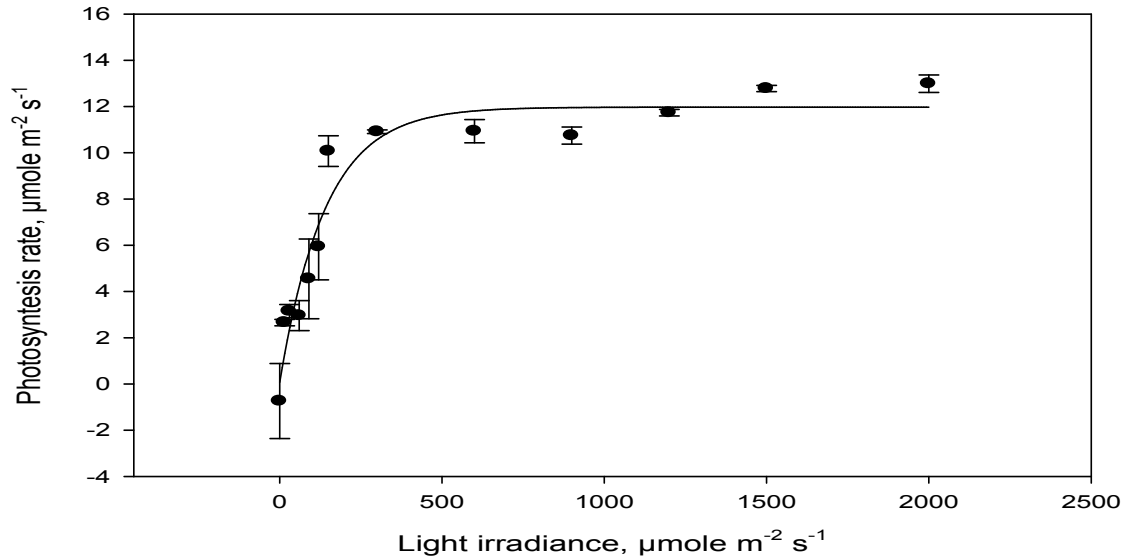


Figure 30: The effect of varying light intensity on the photosynthetic rates in the leaves of *Hymenophyllum denticulatum*. Points show the mean values calculated from 3 readings.

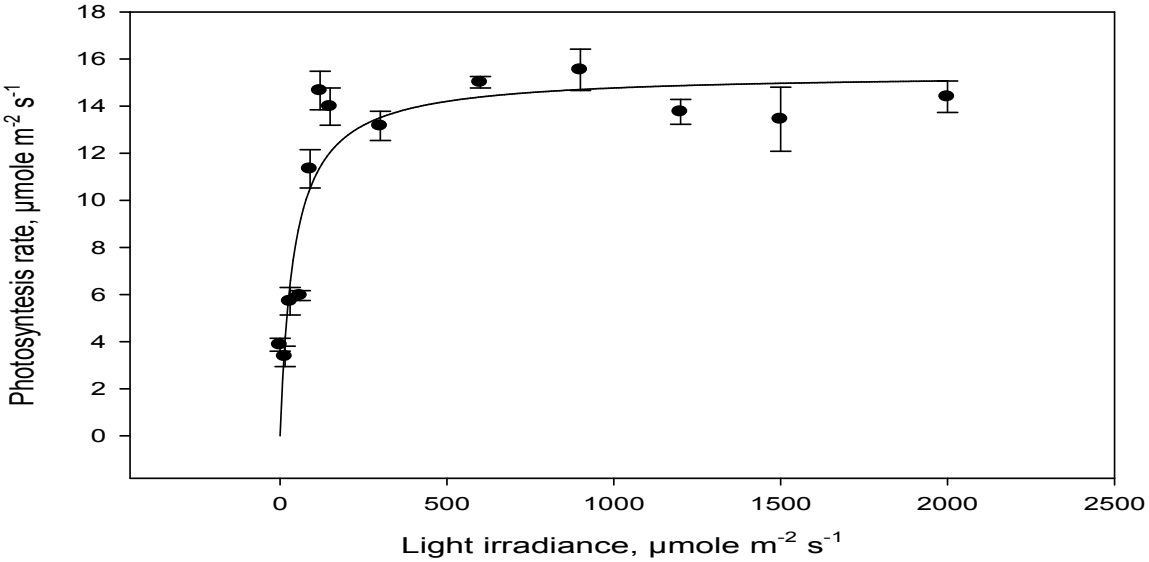


Figure 31: The effect of varying light intensity on the photosynthetic rates in the leaves of *Hymenophyllum exsertum*. Points show the mean values calculated from 3 readings.

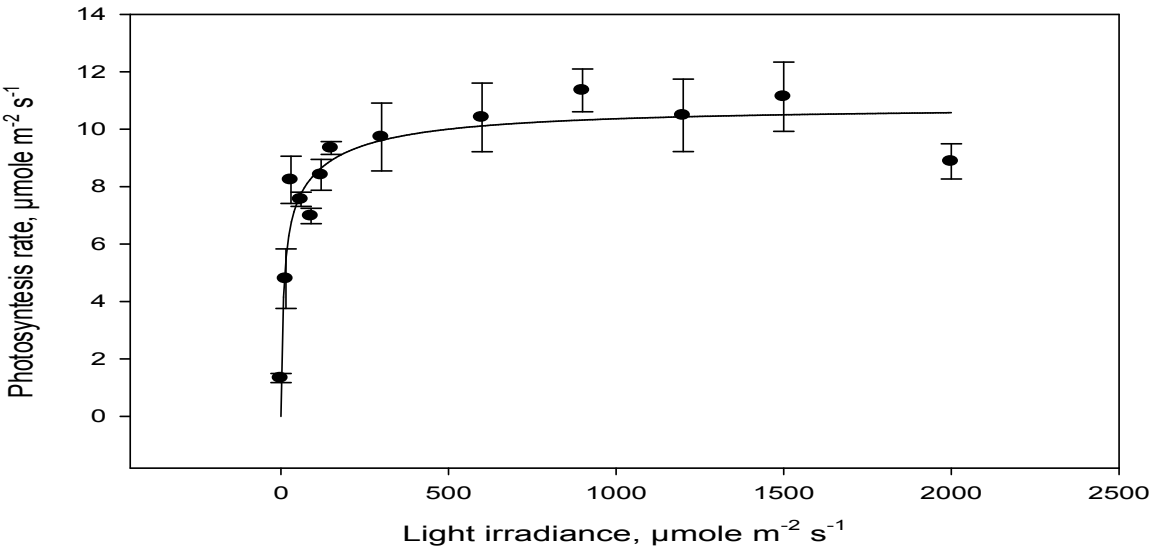


Figure 32: The effect of varying light intensity on the photosynthetic rates in the leaves of *Hymenophyllum serrulatum.* Points show the mean values calculated from 3 readings.

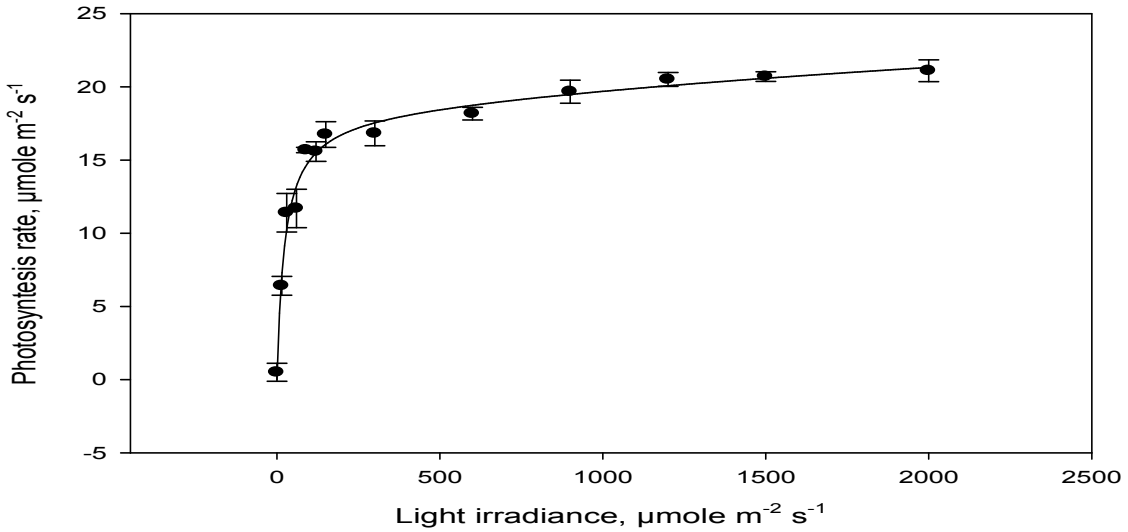


Figure 33: The effect of varying light intensity on the photosynthetic rates in the leaves of *Hymenophyllum acanthoides.* Points show the mean values calculated from 3 readings.

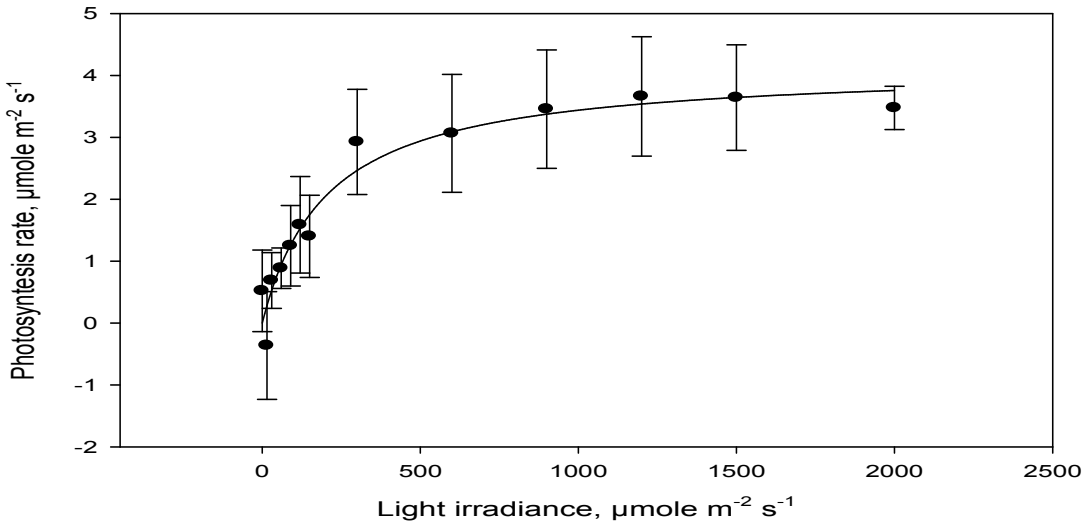


Figure 34: The effect of varying light intensity on the photosynthetic rates in the leaves of *Hymenophyllum blandum.* Points show the mean values calculated from 3 readings.

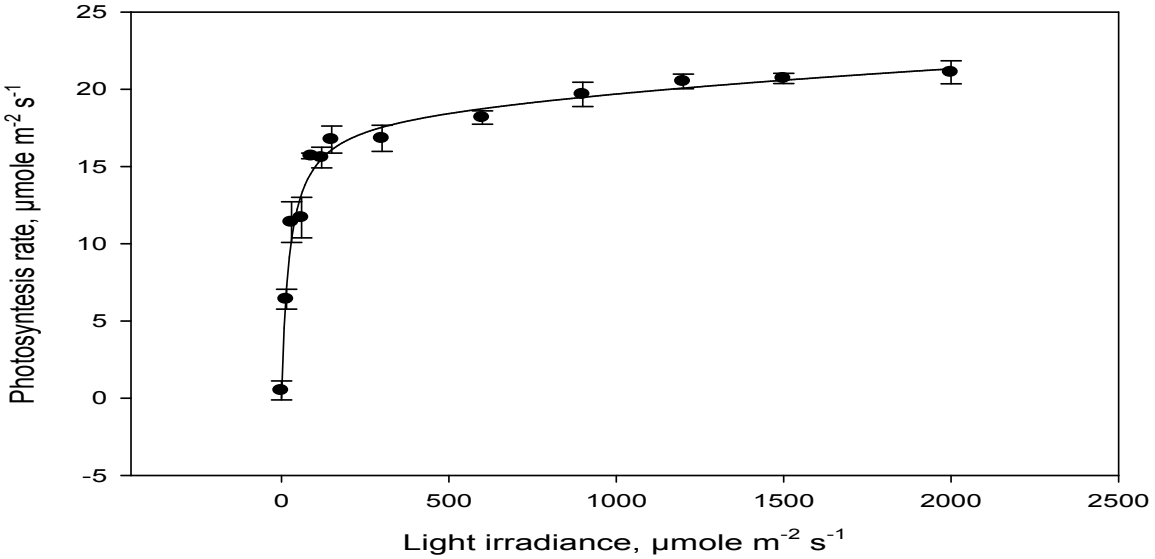


Figure 35: The effect of varying light intensity on the photosynthetic rates in the leaves of *Cephalomanes obscurum.* Points show the mean values calculated from 3 readings.

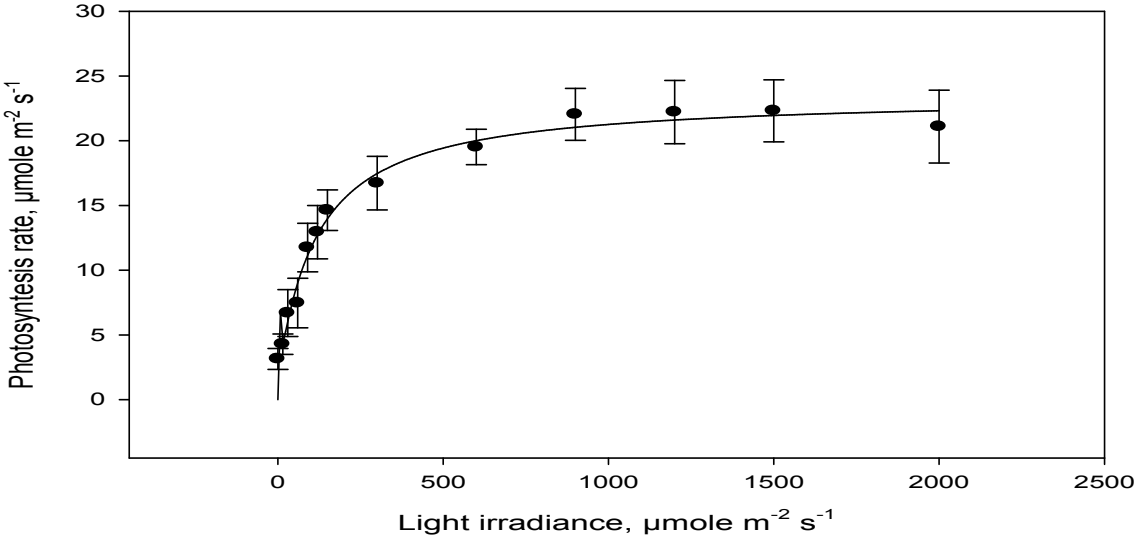


Figure 36. Photosynthetic light response curves of common Malaysian sun fern species, *Dicranopteris linearis.* Each point is an average of 3 readings.

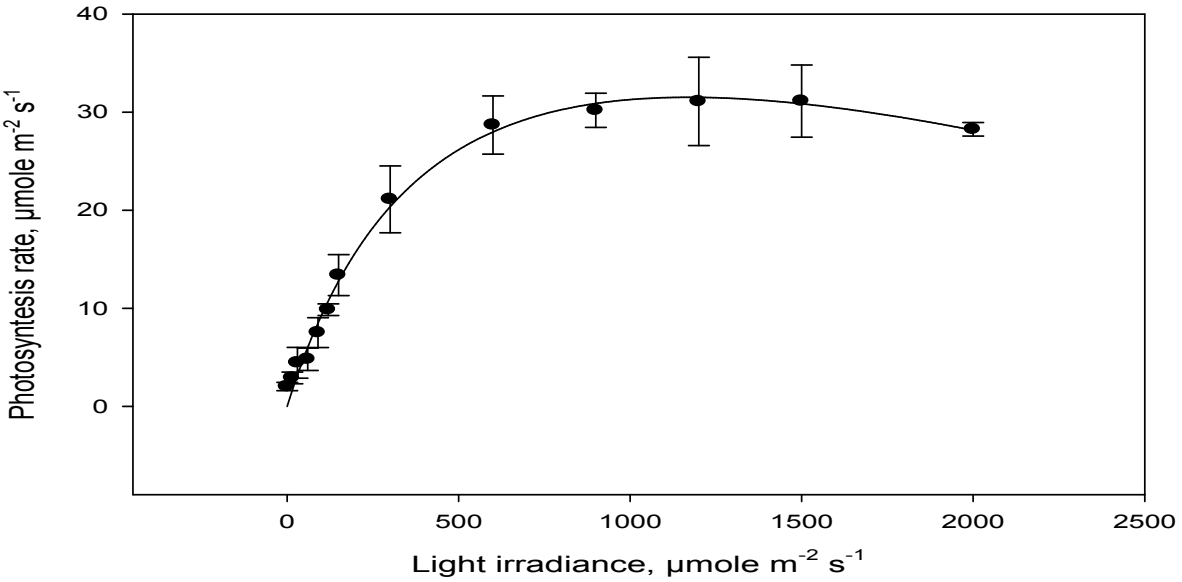


Figure 37. Photosynthetic light response curves of common Malaysian sun fern species, *Nephrolepis biserrata.* Each point is an average of 3 readings.

4.6 Protein expression in Hymenophyllaceae and two sun ferns leaves

Crude extracts prepared from each fern species were electrophoresed on a 12% SDS-polyacrylamide gel to reveal the major protein band (**Figure 38**). Significant differences were detected in the pattern of protein distribution in the extracts, where apparent differences in relative intensities of the bands could be observed. *Nephrolepis biserrata* living in a sunny environment, exhibited a striking protein band compared to the shaded Hymenophyllaceae species. The protein band expressed from this sun plant was thicker than the protein bands that appeared in the Hymenophyllaceae species extract, especially for protein band with molecular weight ranging around 51~62 kDa which is believed to represent the key photosynthetic enzyme, Rubisco. Suprisingly, *H. exsertum, H. acanthoides* and *T. meiofolium* also showed more or less the same in the relative intensities of the band with *Nephrolepis biserrata* compared with *Dicranopteris linearis* in SDS-polyacrylamide gel. A similar pattern of results was also observed in Rubisco protein expression through western blotting (**Figure 39**), where *Nephrolepis biserrata*, *H. acanthoides* and *H. exsertum* expressed highest intensities of RbcL band compared to the other extracts. *H. serrulatum* showed the lowest amount of RbcL expressed.

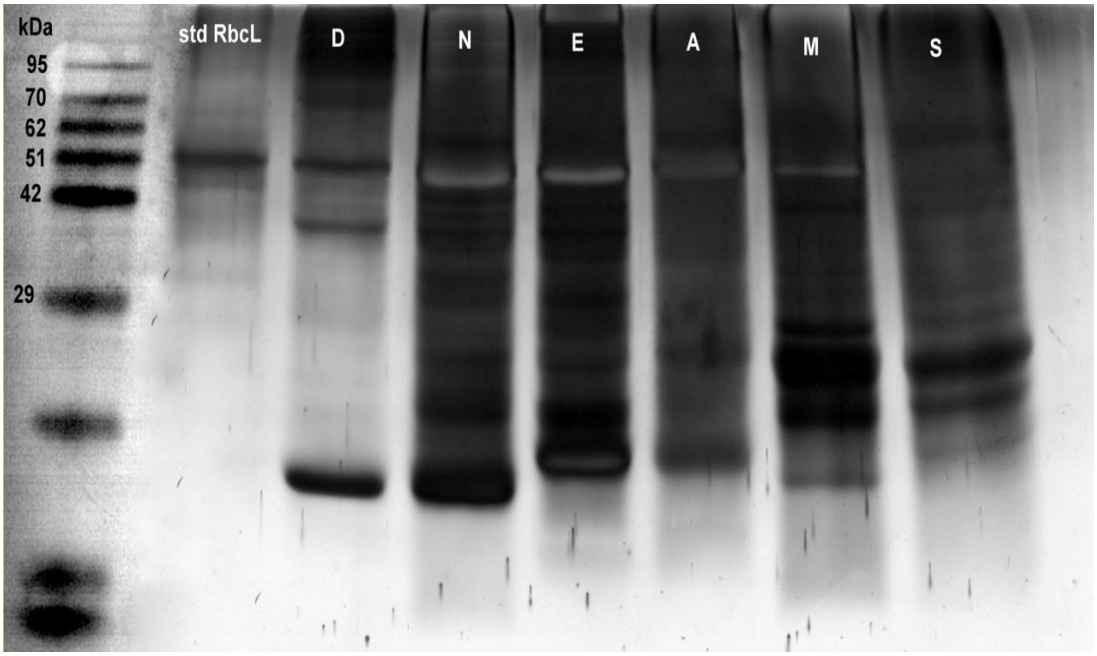


Figure 38. SDS gel electrophoresis of soluble protein from the leaves of two sun ferns and four species of Hymenophyllaceae. Lanes (from left): Molecular mass standard; RbcL standard; D (*Dicranopteris linearis*); N (*Nephrolepis biserrata*); E (*H. exsertum)* ; A (*H. acanthoides*) ; M (*T. meiofolium*) ; S *(H. serrulatum)*; stained with silver (~20ug protein)

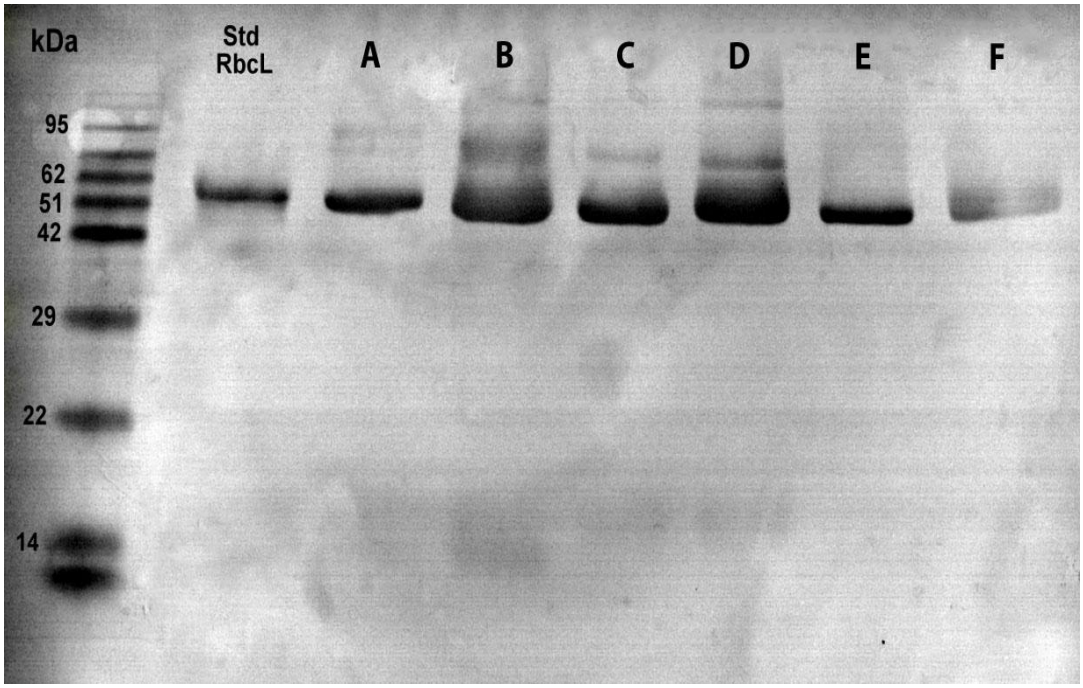


Figure 39. Immunodetection of Rubisco protein in two sun ferns and four *Hymenophyllaceae* species; from the left lane, Standard marker, Standard Rbcl, (A) *Dicranopteris linearis* (sun)*;* (B) *Nephrolepis biserrata* (sun)*;* (C) *Hymenophyllum exsertum;* (D) *Hymenophyllum acanthoides;*(E) *Trichomanes meiofolium;* (F) *Hymenophyllum serrulatum.* Protein enriched (20 µg) samples were separated on a 12.5% SDS-PAGE gel. Following blotting onto a nitrocellulose membrane, the blot was probed with anti-Rubisco polyclonal antibody, HRP conjugated. Purified Rubisco protein standard (10 µg) was used as a marker.

CHAPTER 5

DISCUSSION

As was shown in **Table 6**, the eight species selected for this study, thrived in cool habitats at high elevation with low light irradiances. This was expected as it is well known and documented that the Hymenophyllaceae thrive in damp, moist and shady environments (Dubuisson *et al*, 2003).

Table 7 showed the chlorophyll content expressed on a fresh weight basis was remarkably high in the needle-like dark green leaves of *Trichomanes meiofolium.* The chlorophyll value recorded significantly higher than what has been previously reported for many shade plants. The values of 8.6 mg/g and 6.8 mg/g fresh weight observed for *Trichomanes meiofolium, C. obscorum* and *H. serrulatum* were notably higher than what has been reported in *Teratophyllum rotundifoliatum* (Nasrulhaq-Boyce & Duckett, 1991), *Selaginella* sp. (Hebant & Lee, 1984) and *Pogonatum cirratum* subsp. *macrophyllum* (Nasrulhaq-Boyce *et al.,* 2011). Nevertheless, chlorophyll values in all the eight species studied were high, when compared to previous reports for the ferns *Selaginella wildenowii* (Hebant & Lee, 1984), *Abacopteris multilineata, Christensenia aesculifolia, Tectaria singaporeana* and *Tectaria vasta* (Nasrulhaq-Boyce & Mohamed, 1984) and *Teratophyllum rotundifoliatum* (Nasrulhaq-Boyce & Duckett, 1991), all of which showed values ranging from 2.0 ~ 5.8 mg/g fresh weight. More recent studies on sun and shaded Malaysian *Pogonatum* species showed lower chlorophyll values (Nasrulhaq-Boyce *et al.,* 2011). The high chlorophyll content associated with the low irradiance level in the habitats in which the filmy ferns lived reflects the adaptation to living in a shady environment.

Another observation made was the generally low chlorophyll *a/b* ratios (0.9 to 1.9) in the Hymenophyllaceae leaves compared to the sun ferns (*Dicranopteris linearis* and *Nephrolepis biserrata*), shown in **Table 7**. This is indicative of plants possessing a larger proportion of chl *a/b*-binding light harvesting complexes associated with photosystem II (PSII) and also chloroplasts with more granal thylakoids where the PSII resides. This can also be regarded as an adaptation for shaded plants adapted to living in low light environments (Anderson *et al.,* 1988; Chow *et al.,* 1988; Brach *et al,* 1993; Bjorkman & Demmig-Adams, 1995; Johnson *et al.,* 2000; Marschall & Proctor. 2004; Nasrulhaq-Boyce *et al.,* 2011, Proctor, 2012, Wong *et al.,* 2012). Report from studies on other shade ferns gave higher values ranging from 2.1 to 2.6 (Nasrulhaq-Boyce & Mohamed, 1987). In their study, the sun ferns had higher ratios, between 2.5 to 2.9. The low chlorophyll *a/b* ratios observed in the Hymenophyllaceae species indicate a higher content of chlorophyll *b* in these plants. The higher proportion of chlorophyll *b* will probably enhance their light-absorbing capacity in the wavelength region between the main blue and red bands of the weak diffused light that reaches the forest floor (Bowsher *et al.,*2008). A previous study on *Trichomanes speciosum* reported that the low chl*a/b* ratio observed is accompanied by an increase in the proportion of stacked thylakoids within the chloroplast, in line with other shady plant's adaptation (Johnson *et al.,* 2000). Similarly, Mathew *et al.* (2005) reported the higher chlorophyll *a/b* ratio seen in the sunny fern *Onoclea sensibilis* indicated a smaller light harvesting system (less stacking of thylakoid membranes). In general, plants found in higher light environments, tend to produce a higher chlorophyll *a:b* ratio (Ludlow and Wolf, 1975). In addition, the fern of *Woodsia-montevidensis* also exhibited a similar characteristic as mentioned above, where they showed lower concentrations of total chlorophyll at increasing altitude (Gonzales *et al.*, 1993). All this point to the general view that plants

growing or living in the shade will have a substantially higher chlorophyll content and a higher chlorophyll *a/b* ratio than those plants which normally grow under the bright sunlight.

Generally, plants living in shade environments have larger chloroplasts but lower chloroplast number than plants thriving in an exposed area (Boardman, 1977). Studies on the deep shade fern *Teratophyllum rotundifoliatum*, which possesses large grana stacks showed the highest number of thylakoids per granum, packing stroma of the chloroplast (Nasrulhaq-Boyce & Duckett, 1991). Several other reports have reported similar observations (Hebant & Lee, 1984; Givnish, 1988; Proctor, 2004). It has also been reported that the blue green coloration in the *Trichomanes elegans* is due to the remarkably uniform thickness and the arrangement of the grana in the chloroplast which can be found adjacent to the adaxial wall of the adaxial epidermis (Graham *et al.,* 1993). Study of the extremely deep shade fern of *Trichomanes speciosum* by Makgomol and Sheffield (2001) found that the gametophyte filament cells have a small, spherical or ovoid chloroplast compared with their sporophyte leaf cell, which have much fewer but slightly larger and disc-shaped chloroplasts. These variations enable the plants to make efficient use of light which they need to achieve in order to avoid photosynthetic starvation.

As shown in **Table 9**, the chloroplast number per profile observed in the leaves of the shaded *H. acanthoides, H. serrulatum, H. exsertum, H. javanicum, H. denticulatum,* and *H. blandum* were relatively low (34-63) and the diameter of the chloroplasts were significantly large (5.6-6.1µm), consistent with previous research findings, with the exception of *Cephalomanes obscorum,* which showed an extraordinary cell wall structure with high numbers of chloroplast (138). The latter also had the smallest chloroplast size (4.8µm). Nevertheless, not all shaded plants have

similar anatomical structures. Some plants like *Alocasia macrorrhiza* (Chow *et al.,* 1988) and *Fatsia japonica* (Vidal *et al.,* 1990) exhibit low numbers of chloroplast per cell whilst living in environment of high light irradiance, whilst *Trichomanes meiofolium* (*Hymenophyllaceae*), which grows in shady habitats possesses lamina cells of 100-200 chloroplast per cell, each only 3-6μm in diameter, despite their needle-like structure (Nasrulhaq-Boyce & Duckett, unpublished data).

Generally, shade plants make efficient use of the available photon flux they are able to capture in order to avoid photosynthetic starvation and they do this by having leaf morphology, pigment content and chloroplast structure and size that enable them to achieve it. Recently, Sheue *et al.* (2007) reported a single giant cup-shaped chloroplast, termed a bizonoplast found in the deep-shade spike moss *Selaginella erythropus*. They suggested that the chloroplast structure may have an evolutionary significance in photosynthetic functionality in adaptation to low-light environments. Furthermore, the presence of "Iridoplast", a highly modified plastid in this plant is thought to be associated with iridescent blue color (Lee *et al.,* 1997, 2001). The iridescent blue coloration occurs in plants growing in the extreme shade of tropical rain forest is probably due to multiple layers of cellulose microfibrils in the external cell walls of the adaxial epidermis and the presence of the unusual "Iridoplast" (Sheue *et al.,* 2007).

It is well documented that the amount of soluble protein content or ratio of soluble protein to chlorophyll content in shade plants are considerably lower than the sun species (Boardman, 1977; Givnish, 1988; Nasrulhaq-Boyce & Mohamed, 1987; Nasrulhaq-Boyce *et al.,*2011). This behaviour is due to the sun plants having more protein to enable them to photosynthesize more rapidly and efficiently in sunlit habitats and also because they have less chlorophyll than shade plants. As shown in **Table 8**, the protein content determined by the Lowry, Bradford and BCA assay methods were low

in most of the *Hymenophyllaceae* ferns with values ranging between 0.3-10 mg g^{-1} fresh weight, compared to the sun ferns with values ranging between 12-20 mg g^{-1}. However, *H. serrulatum* exhibited a remarkably high value, above 50 mg g^{-1} fresh weight when determined using the Lowry method. This result is contrary to previous reports for sun and shade plants. There is the possibility that the high protein value via Lowry method could be due to phenolic compounds present in the leaves and extracts which can lead to an overestimation of the protein content using the Lowry method (Lindeboom & Wanasundara, 2006). Previously, there have been reports that interference produced by plant secondary metabolites can cause the reduction of the folin phenol reagent with a resulting increase in colour formation (Jeffrey, 1982). He tested twenty-four compounds using the Lowry method of protein determination, and found that the compounds produced significantly higher values of concentration. Due to these circumstances, the bicinchoninic acid (BCA) protein assay, developed by Smith *et al*. (1985) was used in the determination of the protein content of the Hymenophyllaceae species and the sun ferns. Nevertheless, the protein values for *Hymenophyllaceae serrulatum* was still considerably high (34 mg g^{-1}), eventhough in general, the BCA method is known to decrease sensitivity due to chemical interferences. The BCA protein assay has the same sensitivity as the Lowry method, except that the BCA substitutes for the Folin-Ciocalteau reagent used in the Lowry assay.

All the Hymenophyllaceae species studied in this experiment except for *H. serrulatum* exhibited a lower soluble protein to chlorophyll ratio (**Table 8**) than do in sun fern species (*Dicranopteris linearis* and *Nephrolepis biserrata*). The soluble protein to chlorophyll values was lower than that observed in several shady angiosperm species (Boardman, 1977; Nasrulhaq-Boyce & Mohamed, 1987). The soluble protein in plants usually reflects the concentration of ribulose bisphosphate carboxylase-oxygenase

(Rubisco), a major protein of leaves in higher plants. This protein is one of the light regulated chloroplast photosynthetic enzymes whose activation and probably synthesis also requires light (Rao & Hall, 1994). This may partially account for the lower protein content in plants growing under low light intensities. The results also suggest that the shaded Hymenophyllaceae plants probably invest more of their synthetic capacity for the production of light harvesting assemblies rather than for the synthesis of Rubisco or other soluble proteins or enzymes.

There has been little information on the protein profile of sun and shade adapted plants before this. The morphological and physiological differences observed in these ferns that are adapted to the shaded environment led us to question whether there are any alterations or differences in the protein pattern between the Hymenophyllaceae species and sun-adapted ferns, especially with regard to the key photosynthetic enzyme, Rubisco protein. Protein profiling, employing SDS gel electrophoresis showed clear differences between the protein bands in the Hymenophyllaceae species and the sun fern species, *Nephrolepis biserrata* and *Dicranopteris linearis* (**Figure 38**). As mentioned earlier, sun plants are expected to produce a higher amount of soluble protein as they carry out photosynthesis at a faster rate and one of the key enzymes involved, Rubisco, is a major leaf protein, amounting up to 25-50% of its total protein (Schulze & Caldwell, 1994). *Nephrolepis biserrata* living in a sunny environment, exhibited a striking protein band compared to the shaded Hymenophyllaceae species especially for the protein band with molecular weight ranging around 51~62 kDa which is believed to represent the key photosynthetic enzyme, Rubisco. Suprisingly, *H. exsertum, H. acanthoides* and *T. meiofolium* also showed more or less the same in terms of the relative intensities of the band with *Nephrolepis biserrata* compared with *Dicranopteris linearis* in SDS-polyacrylamide gel. A study of *Solidago virgaurea* (Björkman, 2006)

showed that enzyme extracts from clones native to an exposed area contribute to higher activities of carboxydismutase (ribulose-1,5-diphosphate carboxylase) compared to a shaded habitat. Suprisingly, despite their lower protein content, the shaded *H. exertum, H. acanthoides* and *T. meiofolium* expressed similar amounts of Rubisco even though they come from a shaded environment **(Figure 39)**. However, it must be noted that the exact amount of Rubisco protein in the selected Hymenophyllaceae species were not determined in this study. It can only be assumed that the three Hymenophyllaceae species invest a lot in the production of Rubisco enzyme proteins in order to cope with living in their shaded surroundings.

Chlorophyll fluorescence in *H. denticulatum, T. meiofolium*, and *H. serrulatum* exhibited Fv/Fm ratios or photosynthetic quantum yield values, ranging between 0.73 to 0.81 (**Table 10**). These values are generally slightly lower than what has been recorded for a normal healthy plant which are usually between 0.82 ~0.83 (Peter Horton, personal communication). The low light intensities in which these plants thrive, make it difficult to get an accurate estimate of the maximum quantum yield using fluorescence. Our data suggest the maximum value of the ratio of variable to maximal fluorescence (F_v/F_m) to be around ~0.8. Both F_v and F_m levels increase with increasing chlorophyll content since leaves with more chlorophyll will display a higher intensity of fluorescence although it should not affect the F_v/F_m ratio. Previous studies on *Trichomanes speciosum* showed variable to maximal fluorescence (F_v/F_m) ratios of around 0.75, lower than the F_v/F_m ratios of *T. meiofolium* and *H. denticulatum* studied (Johnson *et al.,* 2000). They suggested that *T. speciosum* possessed a limited ability to quench chlorophyll fluorescence. However, Proctor (2003) reported the Fv/Fm ratios measured in *H. wilsonii* and *H. tunbrigense* was approximately 0.82, which is close to the values obtained for the Malaysian Hymenophyllaceae ferns and *Trichomanes speciosum.*

Recent studies by Wong *et al.* (2012) also reported Fv/Fm values for all dark adapted-fern leaves (*Pyrossia lingus, Asplenium antiquum, Diplazium donianum* and *Archangiopteris somai*) in the region of ~0.8. The lower chlorophyll fluorescence values observed in *H. denticulatum, T. meiofolium, H. javanicum* and *H. serrulatum* probably indicate their limited ability to quench chlorophyll fluorescence. The potential quantum efficiency PSII is reflected in the dark-adapted Fv/Fm values. Thus it is used as a sensitive indicator of plant photosynthetic performance with optimal values 0.83 among all of the plant species (Maxwell & Johnson, 2000). In this study, the lower Fv/Fm values in *Trichomanes meiofolium* (0.77), *H. javanicum* (0.74) and *H. serrulatum* (0.73) could also possibly be due to environmental stress. It has been shown that Fv/Fm will decrease when shade-adapted plants are exposed to high-light stress (Anderson and Aro, 1994).

It has been well documented in higher vascular plants that plants grown under high light intensity show a greater photosynthetic capacity at light saturation than shade plants, but lower rates at low light intensities compared to shade plants (Boardman, 1977; Nasrulhaq-Boyce & Mohamed, 1987; Givnish, 1988; Johnson *et al.* 2000; Proctor, 2012; Wong *et al.,* 2012). Previous studies on filmy ferns *Hymenophyllum wilsonii* and *Hymenophyllum tunbrigense* have shown that saturation of photosynthetic electron flow and CO_2 uptake are generally lower at low irradiances (Proctor, 2003). Furthermore, it has been reported that isolated chloroplasts from sun Malaysian ferns showed greater *in vitro* photochemical activity at saturating irradiance than chloroplasts from shade ferns (Nasrulhaq-Boyce & Mohamed, 1987). They suggested that the greater capacity for electron transport might be attributed to the observed higher level of electron transport carriers, the photosynthetic cytochromes *f*, b_{559}, $b_{563,}$ found in the sun ferns. Marschall and Proctor (2004) studied 39 species of mosses and 16 liverworts for

their ratios of chlorophyll and total carotenoids and light saturation of photosynthetic electron flow and concluded that total chlorophyll, chlorophyll *a:b* and chlorophyll:carotenoids ratios correlated significantly with photosynthetic photon flux density.

The *in vivo* CO_2 assimilation activities in the leaves of the filmy fern revealed low rates consistent with those observed in other ferns and bryophytes. Recent studies by Proctor (2012) showed the photosynthetic rate of Hymenophyllaceae species from Trinidad, Venezuela and New Zealand saturated at low light intensities, where all four of the shade-adapted species had $PPFD_{95\%} \leq 51$ µmole m^{-2} s^{-1}. Similarly, another study on four fern species (*Pyrrosia lingus, Asplenium antiquum, Diplazium donianum* and *Archangiopteris somai*) by Wong *et al.* (2012) showed low gross photosynthetic rate (P_g) when the dark-adapted leaves were exposed to 500- 2000 µmole m^{-2} s^{-1}. In this study the shade-adapted *H. blandum* recorded the lowest photo-assimilatory rates (~3 µmol CO_2 m^{-2} s^{-1}) compared to *H. javanicum* (~5 µmol CO_2 m^{-2} s^{-1}), *H. serrulatum* (~9 µmol CO_2 m^{-2} s^{-1}), *Hymenophyllum denticulatum* (~10 µmol CO_2 m^{-2} s^{-1}), and *Trichomanes meiofolium* (~11 µmol CO_2 m^{-2} s^{-1}). Meanwhile, *H. exsertum, H. acanthoides* and *Cephalomanes obscorum* exhibit higher photosynthesis rates, approximately 14 to 17 µmol CO_2 m^{-2} s^{-1}. These *Hymenophyllaceae* species showed optimal photosynthesis at light intensities between 100 to 150 µmol/m^2/s, the highest light intensity to which they are normally exposed to in their natural environment, except for *H. blandum* which saturated at around 300-400 µmole $m^{-2}s^{-1}$ (**Figure 28-35**).

The sun-adapted fern *Dicranopteris linearis* and *Nephrolepis biserrata,* recorded higher photosynthetic rates of 22 µmol CO_2 m^{-2} s^{-1} and 30 µmol CO_2 m^{-2} s^{-}, respectively (**Figure 36-37**). These findings are consistent with previous results, where plausible reasons for the different responses include the higher chlorophyll content in the shade

leaves compared to the sun leaves (Boardman, 1977; Givnish, 1988; Alfredo *et al.,* 2010; Huang *et al.,* 2011) and the adaptability of the *Hymenophyllaceae* leaves to utilize periodic sunflecks (Johnson *et al.,* 2000; Proctor, 2003; Proctor, 2012; Wong *et al.,* 2012). This should not be suprising since the Hymenophyllaceae species in their natural habitat live in the shade and thus need low light intensities for them to attain saturation point. Besides that, the observation of a positive photosynthetic activity at zero light irradiance occurred possibly due to the presence of sufficient amounts of NADPH and ATP in the chloroplasts, which is normally provided from the light reactions in the thylakoid membranes that was enough to drive CO_2 fixation and the reduction processes in the Calvin cycle. It has been reported that the maximum rates of carbon dioxide uptake of shade plants fall within the range of 0.6-5.2 μmol $m^{-2}s^{-1}$, but the saturation light intensities recorded were slightly higher (Hietz and Briones, 2001). While, Johnson *et al* (2000) has reported the measurement of gas exchange for *Trichomanes speciosum* have optimal photosynthesis at similarly low light intensities, between 5-10 μmole CO_2 $m^{-2}s^{-1}$. The photosynthetic rate differences in the filmy ferns studied could be due to the variation pattern of adaptation in these plants.

Studies on *Hymenophyllum willsoonii* and *Hymenophyllum tunbrigense* by Proctor (2003) has shown that *H. willsonii* has somewhat a higher light requirement than *H. tumbrigense,* even though both filmy ferns are well adapted in lower light levels. The same situation is probably observed in *Cephalomanes obscorum, H. exsertum* and *H. acanthoides,* where they may require a much higher light intensity compared to the other *Hymenophyllaceae* species studied. The other plausible reason is that it could be related to their morphological characteristics of leaf, chloroplast number and size, as mentioned before. The higher number of chloroplasts in *Cephalomanes obscorum* especially, could possibly enhance their light energy capture in order to adapt

to low light environment. Proctor (2012) believed that filmy ferns (Hymenophyllaceae) are a rare example of species of an evolutionary shift of adaptive strategy from typical vascular plant adaptation with an integrated package adapted to more or less constantly shaded humid environments where its photosynthesis saturates at low irradiance and it generally has low levels of dessication tolerance. This study also suggests that the lower photo assimilatory rates found in the Hymenophyllaceae species are possibly due to a lower stomatal frequency, although the comparisons of stomatal number were not tested here. It has been reported that the stomatal number and density was higher in the sun ferns than in the shade species (Rabiah, 1983).

Generally, the sun ferns need relatively higher rates of transpiration to reduce their leaf temperature. This is because the atmospheric temperature of the habitat of sun ferns is always higher than the habitats of the shade ferns. Thus, at higher temperatures, photosynthesis, transpiration and the gaseous exchange (CO_2, O_2 and water vapour) occur at a faster rate. The faster exchange of gases requires, a greater number of stomata per unit leaf area through which the movement of gases can occur. The reverse applies to the shade ferns. So, it is possible that the lower CO_2 assimilation rate in most of the shaded *Hymenophyllaceae* could be related to the reasons mentioned above. Atala *et al* (2012) reported that the shade-tolerant ferns species, *Blechnum mochaenum* depend partially on their stomata traits. They reported a lower stomatal density, stomatal index and higher stomatal size in the leaves of the shaded *B. mochaenum* compared to their sun counterparts (*Blechnum chilense*). Besides the stomatal factor, previous studies have also suggested that the hydraulic adjustment of fern fronds is a key component for the adaptation of pteridophytes to contrasting light habitats (Lo Gullo *et al*, 2010). Besides the biochemical adaptation mentioned previously, it appeared that some of the gametophytes of *Hymenophyllaceae* species also show a morphological variation that

make them well adapted to shaded habitats, as in the *Trichomanes speciosum* (Johnson *et al,* 2000).

Thus, this study has shown that the Hymenophyllaceae species are able to adapt well to their shaded environments and possess the ability to make efficient use of what little light is available to them for photosynthesis and growth. It appears that the adjustment of the photosynthetic capacity and performance to light availability is an important mechanism in these shaded Hymenophyllaceae, in order for them to thrive and survived in humid and shaded area. It is clear that the filmy ferns are a successful group of plants in the shaded habitats.

CHAPTER 6

CONCLUSIONS

The results from the comparative studies made between eight species of shade Hymenophyllaceae and two sun ferns; *Dicranopteris linearis* and *Nephrolepis biserrata* have shown that the shade ferns species had high chlorophyll content based on fresh weight basis than the sun ferns. The shade ferns species also exhibited low chlorophyll *a/b* ratios. The soluble protein to total chlorophyll ratio in shade filmy ferns were lower than what has been reported earlier for shade ferns. In the protein profiling observation, *Nephrolepis biserrata* living in a sunny environment, exhibited a striking protein band compared to the shaded Hymenophyllaceae species. Similar results were also observed in Rubisco protein expression through western blotting, that may account for the higher protein content in plants growing under high light intensities.

Light microscopy observations showed that *Cephalomanes obscorum* had the highest number of chloroplasts per cell profile and smallest in size amongst the *Hymenophyllaceaec* species. Nevertheless chloroplast number in *H. acanthoides, H. serrulatum, H. exsertum, H. javanicum, H. denticulatum* and *H. blandum* were relatively low and the cells were closely packed with chloroplasts, similar with what has been documented previously for shaded plants. This supports the widely held view that shade plants make efficient use of the available photon flux they are able to capture in order to avoid photosynthetic starvation and they do this by having leaf morphology, pigment content and chloroplast structure and size that enable them to achieve it.

Chlorophyll fluorescence in *H. denticulatum, T. meiofolium* and *H. serrulatum* exhibited lower Fv/Fm or photosynthetic quantum yield values, ranging between 0.73 to 0.81, probably due to their limited ability to quench chlorophyll fluorescence. *In vivo* light saturation curve experiments showed that saturation point for whole photosynthetic rates was achieved at low light intensities below 150 μ mole $m^{-2}s^{-1}$, , except for *H. blandum* which saturated at around 300-400 μmole $m^{-2}s^{-1}$. The two sun ferns (*Dicranopteris linearis* and *Nephrolepis biserrata*) showed optimal photosynthesis at higher light irradiance (~600μmole $m^{-2}s^{-1}$) than what was observed in shade Hymenophyllaceae species. These photosynthetic characteristic reflects a plant growing in the deep shade maximizing use of very little available light. Further studies on other photosynthetic parameters such as stomatal conductance, transpiration rate and activities of certain key photosynthetic enzymes might shed more light on its photosynthetic behavior.

REFERENCES

Abrams, M. D., Kloeppel, B. D. and Kubiske, M. E. (1992). Ecophysiological and morphological response to shade and drought in two contrasting ecotypes of *Prunus serotina*. *Tree Physiol*, 10(4): 343-355.

Anderson, J. M., Chow, W. S., Goodchild, D. J. (1988). Thylakoid membrane organisation in sun/shade acclimation. *Australian Journal of Plant Physiology,* 15: 11–26.

Anderson, J. M. and Aro, E. M. (1994). Grana stacking and protection of photosystem II in thylakoid membranes of higher plant leaves under sustained high irradiance: An hypothesis. *Photosynthesis Research,* 41: 315-326.

Alfredo O. S., Hernandez C., Rafael E. C, Leon A.B. and Luis J. Corcuera (2010). Differences in light usage among three fern species of genus *Blechnum* of contrasting ecological breadth in a forest light gradient. *Ecological Research,* 25: 273-281.

Arinawati, A. (1995). Studies on some photosynthetic characteristics of two *Teratophyllum* species with emphasis on *Teratophyllum rotundifoliatum* (R.Bonap.) Holtt. (BSc.Hons.) Thesis, Dept. of Bot., U.M. (unpublished).

Arnon, D. L. (1949). A copper enzyme is isolated chloroplast polyphenol oxidase in *B. vulgaries*. *Plant Physiol,* 24: 1-15.

Atala, C., Saldana, A. and Navarrete, E. (2012). Stomatal frequency and gas exchange differs in two *Blechnum* species (Pteridophyta, Blechnaceae) with contrasting ecological breadth. *Gayana Botanica,* 69 : 161-166.

Bhattacharya, A., Sood, P. and Citovsky, V. (2010). The roles of plant phenolics in defence and communication during Agrobacterium and Rhizobium infection. *Molecular Plant Pathology,* 5: 705-719

Björkman, O. and Holmgren, P. (1966). Photosynthetic adaptation to light intensity in plants native to shaded and exposed habitats. *Plant Physiology,* 19: 854-889.

Björkman, O. (1967). Carboxydismutase activity in relation to light-saturated rate of photosynthesis in plants from exposed and shaded habitats. *Carneige Inst. Washington Yearb.* 65: 454-459.

Björkman, O. (1968). Further studies on differentiation of photosyntethic properties in sun and shade ecotypes of *Solidago virgaurea. Plant Physiology*, 21: 84-99.

Björkman, O. (1987). Response to different quantum flux densities. *In* Encyclopedia of Plant Physiology, New Series, Vol. 12A. Eds O. L. Lange, P. S. Nobel, C. B. Osmond and H. Ziegler. Springer-Verlag, Berlin, pp 57-107.

Björkman, O. and Demmig, B. (1987). Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77K among vascular plants of diverse origins. *Planta*, 170: 489-504.

Björkman, O. and Demmig-Adams, B. (1995). Regulation of photosynthetic light energy capture, conversion and dissipation in leaves of higher plants. In: Schulze E-D, Caldwell MM, editors. *Ecophysiology of photosynthesis,* 100: 7–47.

Björkman, O. (2006). Further studies on differentiation of photosynthetic properties in sun ans shade ecotypes of *Solidago virgaurea*. *Physiologia Plantarum,* 21:84-99.

Boardman, N. K. (1977). Comparative photosynthesis of sun and shade plants. *Annual Review of Plant Physiology,* 28: 355–377.

Browsher, C., Steer, M. and Tobin, A. (2008). Plant Biochemistry (1st ed.) Garland Science.

Bohning, R. H., Burnside, C. A. (1956). The effect of light intensity on rate of apparent photosynthesis in leaves of sun and shade plants. *American Journal of Botany,* 43: 556–561.

Brach, A. R., McNaughton, S. J. and Raynal, D. J. (1993). Photosynthetic adaptibility of two fern species of a Northern Hardwood Forest. *American Fern journal,* 83: 47-53.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,* 72: 248–254.

Burnside, C. A., Bohning, R. H. (1957). The effect of prolonged shading on the light saturation curves of apparent photosynthesis in sun plants. *Plant Physiol,* 32: 61–63.

Chow, W. S., Qian, L., Goodchild, D. J., Anderson, J. M. (1988). Photosynthetic acclimation of *Alocasia macrorrhiza* (L.) G. Don to growth irradiance: Structure, function and coposition of chloroplast. *Australian Journal of Plant Physiology,* 15: 107–122.

Demmig, B., Winter, K. (1988). Characterisation of three components of non-photochemical fluorescence quenching and their response to photoinhibition. *Australian Journal of Plant Physiology,* 15: 163–177.

DiCristina, K. and M. Germino. (2006). Correlation of neighbourhood relationships, carbon assimilation, and water status of sagebrush seedlings establishing after fire. *Western North American Naturalist*. 66(4): 441-449.

Dubuisson, J. Y., Hennequin, S., Rakotondrainibe, F. and Schneider, H. (2003). Ecological diversity and adaptive tendencies in the tropical fern *Trichomanes* L. (Hymenophyllaceae) with special reference to climbing and epiphytic habits. *Botanical Journal of the Linnean Society,* 142 (1): 41–63.

Ebihara, A., Hennequin, S., Ito, M., Iwatsuki, K. and Dubuisson, J. Y. (2006). Phylogenetic systematics and evolution of the genus *Hymenophyllum (Hymenophyllaceae: Pteridophyta). Fern Gazette,* 17(5): 247-257.

Evans, J. R. (1988). Acclimation by the thylakoid membranes to growth irradiance and the partitioning of nitrogen between soluble and thylakoid proteins. *Australian Journal of Plant Physiology,* 15: 93–106.

Fereira, J. F. and Janick, J. (1996). Immuno quantitative analysis of aetermisinin from *Artemisia annua* using polyclonal antibodies. *Phytochemistry,* 41(1): 97-104.

Givnish, T. J. (1988). Adaptation to sun and shade: A whole-plant perspective. *Plant Physiol*, 15: 63–92.

Gonzalez, J. A., de Riera, M. Q. and de Israilev, L. A. (1993). "Chlorophyll Concentration and Flavonoids in the Fern Woodsia-Montevidensis in Different Light Regimes at 2 Altitudes in Northwestern Argentina." *Acta Oecologica-International Journal of Ecology*, 14(6): 839-846.

Graham, R. M., Lee, D. W. and Norstog, K. (1993). "Physical and Ultrastructural Basis of Blue Leaf Iridescence in 2 Neotropical Ferns." *American Journal of Botany*, 80(2): 198-203.

Hall, D. O. and Rao, K. K. (1994). *Photosynthesis*. Cambridge University Press.

Hebant, C. and Lee, D. W. (1984). Ultrastructure basis and development control of blue iridescence in *Sellaginella* leaves. *American Journal of Botany,* 71: 216–219.

Heukshoven, J. and Dernick, R. (1986). Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis*, 6 : 103-112.

Hew, C. S. and Wong, S. C. (1974). Photosyntehsis and respiration of ferns in relation to their habitat. *Amrican Fern Journal*, 64: 40-48.

Hietz, P. and O. Briones (2001). "Photosynthesis, chlorophyll fluorescence and within-canopy distribution of epiphytic ferns in a Mexican cloud forest." *Plant Biology*, 3(3): 279-287.

Huang, D., Wu, L., Chen, J. R., and Dong, L. (2011). Morphological plasticity, photosynthesis and chlorophyll fluorescence of Athyrium pachyphlebium at different shade levels. *Photosynthetica,* 49(4): 611–618.

Jeffrey, L. S. (1982). Plant constituents interfering with the Lowry method of protein determination. *Proceedings of the Oklahoma Academy of Science,* 62:80-83.

Johnson, G. N., Rumsey, F. J., Headley, A. D., Sheffield, E. (2000). Adaptations to extreme low light in the fern *Trichomanes speciosum. New Phytologist,* 148: 423–431.

Kobza, J. and Seeman, J. R. (1988). Mechanisam for light-dependent regulation of ribbulose-1,5-bisphosphate carboxylase activity and photosynthesis in intact leaves. *Proc Natl Acad USA,* 85(11): 3815-3819.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227: 680-685.

Lee, D. W., Bone, R. A., Tarsis, S. L. and Stroch, D. (1990). Correlates of leaf optical properties in tropical forest sun and extreme- shade plants. *American Journal of Botany*, 77: 370-380.

Lee, D. W. (1997). Iridescent blue plants. *American Science,* 85: 56-73.

Lee, D. W. (2001). Leaf color in tropical plants: some progress and much mystery. *Malayan Nature Journal,* 55: 117-131.

Lindeboom, N. and Wanasundara, P. K. J. P. D. (2006). Interference of phenolic compounds in *Brassica napus*, *Brassica sinapis alba* seed extracts with the Lowry protein assay. *Food chemistry,* 104: 30-38.

Lo Gullo, M. A., Raimondo, F., Crisafulli, A., Salleo, S. and Nardini, A. (2010). Leaf hydraulic architecture and water relations of three ferns from contrasting light habitats. *Functional Plant Biology,* 37: 566-574.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951). Protein measurement with the Folin-Phenol reagents. *The Journal of Biological Chemistry,* 193: 265–275.

Ludlow, C. J. and Wolf, F. T. (1975). Photosynthesis and respiration rates of ferns. *American Fern Journal,* 65: 43-48.

Makgomol, K. and Sheffield, E. (2001). Gametophyte morphology and ultrastructure of the extremely deep shade fern *Trichomanes speciosum. New Phytologist,* 151: 243-253.

Malkin, R. and Niyogi, K. (2000). Photosynthesis. In: Buchanan, B. B., Gruissem, W., and Jones, R., eds. (2000) *Biochemistry and Molecular Biology of Plants*. American Society of Plant Physiologists, Rockville, M. D., pp. 575-577.

Mathew, W. R., Joshua, P. S., Xu, B., Cunkelman, A. and Ronald, A. B. (2005). A comparison of physiological and morphological properties of deciduous and wintergreen ferns in Sounthern Pennsylvania. *American Fern Journal, 95: 45-56.*

Marschall, M. and Proctor, M. C. F. (2004). Are Bryophytes Shade Plants? Photosynthetic Light Responses and Proportions of Chlorophyll *a*, Chlorophyll *b* and Total Carotenoids. *Annals of Botany,* 94: 593–603.

Mazwell, K., Marrison, J. L., Leech, R. M., Griffiths, H. and Horton, P. (1999). Chloroplast acclimation in leaves of Guzmania monostachia in response to high light. *Plant Physiology*, 121(1): 89-96.

Maxwell, K. and Johnson, G. N. (2000). Chlorophyll fluorescence-a practical guide. *Journal of Experimental Botany,* 51: 659-668.

Michalak, A. (2006). Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. *Polish Journal of Environmental Study,* 15:523-530.

Nasrulhaq-Boyce, A. and Mohamed, M. A. H. (1987). Photosynthetic and respiratory characteristics of Malayan sun and shade ferns. *New Phytologist 105*: 81–88.

Nasrulhaq-Boyce, A. and Duckett, J. G. (1991). Dimorphic epidermal cell chloroplasts in the mesophyll-less leaves of an extreme shade tropical fern, *Teratophyllum rotundifoliatum* (R.Bonap.) Holtt.: a light and electron microscope study. *New Phytologist,* 119: 433–44.

Nasrulhaq-Boyce, A., Mohamed, M. A. H., Lim, A. L., Barakbah, S. S., Yong, K.T. and Nor, D. M. (2011). Comparative morphological and photosynthetic studies on three Malaysian species of *Pogonatum* from habitats of varying light irradiance. *Journal of Bryology,* 33:35–41.

Osmond, C. B., Oja, V. and Laisk, A. (1988). Regulation of carboxylation and photosynthetic oscillations during sun-shade acclimation in *Helianthus annuus* measured with a rapid-response gas exchange system. *Australian Journal of Plant Physiology* 15: 239–251.

Park, R. B. and Sane, P. V. (1971). Distribution of function and structure in chloroplast lamellae. *Annual Review Plant Physiol,* 22: 395-430.

Parris, B. S. and Latiff, A. (1997). Towards a pteridophyte flora of Malaysia: a provisional checklist of taxa. *Malayan Nature Journal*, 50: 235-280.

Proctor, M. C. F. (2003). Comparative ecophysiological measurements on the light responses, water relations and dessiccation tolerance of the filmy ferns *Hymenophyllum wilsonii* Hook. and *H. tunbrigense* (L.) Smith. *Annals of Botany*, 91: 717–727.

Proctor, M. C. F. (2005). Why do Polytrichaceae have lamellae? *Journal of Bryology,* 27: 221-229.

Proctor, M. C. F. (2012). Light and desiccation responses of some Hymenophyllaceae (filmy ferns) from Trinidad, Venezuela and New Zealand: poikilohydry in a light-limited but low evaporation ecological niche. *Annals of Botany* 109(5): 1019–1026.

Rabiah, B. (1983). Comparative photosynthetic characteristics in some sun and shade loving Malaysian ferns. Thesis, Dept. of Bot., University Malaya (unpublished).

Ralph, P. J. and Gademan, R. (2005). Rapid light curves: A powerful tool to assess photosynthetic activity. *Aquatic Botany,* 82: 222-237.

Rao, K. K. and Hall, D. O. (1994). *Photosynthesis* (5th ed.). Cambridge University, Press, Boca Raton, FL.

Salisbury, F. B. and Ross, C. W. (1992). Plant Physiology. Belmont, CA: Wadsworth. pp. 357-407, 531-548.

Saldana , A. O, Hernandez, C., Coopman, R. E., Bravo, L. A. and Corcuera, L. J. (2010). Differences in light usage among three ferns species of genus *Blechnum* of contrasting ecological breadth in a forest light gradient. *Ecological Research,* 25: 273-281.

Sheue, C. R., Sarafis, V., Kiew, S., Liu, H. Y., Salino, A., Kuo-Huang, L. L., Kiew, R., Yang, Y. P., Tsia, C. C., Lin, C. H., Yong, J. W. H. and Maurice, S. B. K. (2007). Bizonoplast, a unique chloroplast in the epidermal cells of microphylis in the shade plant *Selaginella erythropus* (Selaginellaceae). *American Journal of Botany*, 94: 1922-1929.

Schulze, E. D. and Caldwell, M. M. (1994). Overview: Perspectives in Ecophysiological Research of photosynthesis. In: Schulze ED, Caldwell MM, editors. Ecophysiology of Photosynthesis. Berlin: Springer, pp. 553-564.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem*, 150(1): 76–85.

Vidal, D., Griera, E., Marin, P. and Sabido, J. (1990). Anatomical and physiological acclimation of *Fatsia japonica* leaves to irradiance. *American Journal of Botany*, 77(9): 1149-1158.

Wong, S. L., Chen C. W., Huang, H. W. and Weng, J. H. (2012). Using combined measurements for comparison of light induction of stomatal conductance, electron transport rate and CO_2 fixation in woody and fern species adapted to different light regimes. *Tree Physiology,* 32: 535-544.

Table 11: Chlorophyll Content (Chl *a,b, a/b*) Of *Hymenophyllaceae* Species And Sun Ferns

	SPECIES	R	WEIGHT,G	A645	A663	TOTAL CHL	CHL *A*	CHL *B*	*A/B*
1	*H. serrulatum*	1	0.014	0.177	0.371	4.68	3.03	1.66	1.83
		2	0.015	0.201	0.423	4.97	3.22	1.75	1.84
		3	0.019	0.637	1.369	12.55	8.25	4.31	1.91
		4	0.035	0.297	0.651	3.21	2.13	1.07	1.99
		5	0.029	0.475	1.027	6.15	4.06	2.09	1.94
					MEAN	6.312	4.138	2.176	1.902
					SD	3.640937	2.398806	1.248231	0.067602
					SE	1.628276	1.072779	0.558226	0.030232
2	*H. javanicum*	1	0.023	0.347	0.713	5.53	3.53	2	1.77
		2	0.039	0.538	1.061	4.97	3.08	1.89	1.63
		3	0.035	0.282	0.542	2.87	1.75	1.12	1.56
		4	0.0361	0.475	0.969	4.81	3.06	1.76	1.74
		5	0.032	0.446	0.927	5.14	3.3	1.84	1.79
					MEAN	4.664	2.944	1.722	1.698
					SD	1.038017	0.694212	0.347592	0.098843
					SE	0.464215	0.310461	0.155448	0.044204

3	*H. acanthoides*	1	0.017	0.265	0.566	5.82	3.81	2.01	1.9
		2	0.014	0.191	0.409	5.1	3.34	1.76	1.9
		3	0.0376	0.15	0.319	1.49	0.97	0.52	1.87
		4	0.0368	0.382	0.818	3.88	2.54	1.34	1.9
		5	0.0308	0.215	0.458	2.6	1.7	0.9	1.89
					MEAN	3.778	2.472	1.306	1.892
					SD	1.670029	1.095474	0.574557	0.012293
					SE	0.792164	0.519629	0.272536	0.005831
4	*H. exsertum*	1	0.0327	0.278	0.527	3.01	1.82	1.19	1.53
		2	0.0326	0.349	0.673	3.82	2.33	1.49	1.56
		3	0.0362	0.275	0.585	2.83	1.85	0.98	1.89
		4	0.0332	0.269	0.575	3.03	1.98	1.04	1.9
		5	0.0345	0.45	0.961	4.87	3.19	1.68	1.9
					MEAN	3.512	2.234	1.276	1.756
					SD	0.849776	0.571516	0.299883	0.192951
					SE	0.380032	0.25559	0.134112	0.08629
5	*H. blandum*	1	0.0339	0.447	0.888	4.76	2.97	2.31	1.29
		2	0.0345	0.42	0.826	4.38	2.71	1.67	1.62
		3	0.0346	0.253	0.544	2.74	1.8	1.25	1.44
		4	0.0394	0.256	0.559	2.45	1.63	0.82	1.99
		5	0.0335	0.2	0.435	2.25	1.49	0.76	1.96

					MEAN	3.316	2.12	1.362	1.66
					SD	1.165689	0.672681	0.644647	0.310564
					SE	0.521312	0.300832	0.288295	0.138888
6	*H. denticulatum*	1	0.009	0.141	0.31	5.93	3.95	1.96	2.01
		2	0.012	0.203	0.442	6.37	4.22	2.15	1.96
		3	0.011	0.165	0.359	5.65	3.74	1.91	1.96
		4	0.014	0.207	0.45	5.56	3.68	1.88	1.96
		5	0.014	0.202	0.435	5.41	3.56	1.85	1.92
					MEAN	5.784	3.83	1.95	1.962
					SD	0.378391	0.259808	0.118954	0.031937
					SE	0.169222	0.11619	0.053198	0.014283
7	*T. meiofolium*	1	0.053	1.125	2.293	7.76	4.92	2.84	1.73
		2	0.039	0.952	1.984	9.01	5.8	3.21	1.81
		3	0.029	0.642	1.364	8.24	5.38	2.87	1.87
		4	0.033	0.787	1.653	8.83	5.72	3.12	1.83
		5	0.036	0.912	1.895	9.34	6	3.34	1.8
					MEAN	8.636	5.564	3.076	1.808
					SD	0.631926	0.423887	0.216633	0.051186
					SE	0.282606	0.189568	0.096881	0.022891
8	*C. obscorum*	1	0.0105	0.265	0.38	8	3.92	4.09	0.96

		2	0.0131	0.24	0.334	5.75	2.75	3	0.92
		3	0.0102	0.211	0.267	6.28	3.53	3.51	1.01
		4	0.0131	0.279	0.41	6.81	3.4	3.41	1
		5	0.0093	0.213	0.274	6.99	3.13	3.87	0.81
					MEAN	6.766	3.346	3.576	0.94
					SD	0.843107	0.438212	0.422587	0.080932
					SE	0.377049	0.195974	0.188987	0.036194
	SUN FERNS					T	A	B	A/B
1	*Dicranopteris*	1	0.0311	0.205	0.485	2.582	1.803	0.78	2.31
	linearis	2	0.0323	0.202	0.484	2.465	1.735	0.731	2.37
		3	0.0301	0.22	0.517	2.854	1.985	0.87	2.28
		4	0.0314	0.149	0.35	1.852	1.288	0.565	2.28
		5	0.0326	0.26	0.611	3.114	2.166	0.949	2.28
					MEAN	2.5734	1.7954	0.779	2.304
					SD	0.475191	0.329565	0.146015	0.039115
					SE	0.212512	0.147386	0.0653	0.017493
2	*Nephrolepis*	1	0.0313	0.121	0.29	1.524	1.073	0.452	2.374
	biserrata	2	0.033	0.124	0.31	1.512	1.092	0.421	2.594
		3	0.0305	0.103	0.261	1.368	0.996	0.373	2.67
		4	0.0313	0.123	0.308	1.583	1.144	0.439	2.606
		5	0.0316	0.176	0.439	2.239	1.615	0.625	2.584

					MEAN	1.6452	1.184	0.462	2.5656
					SD	0.341225	0.246724	0.095917	0.112235
					SE	0.152601	0.110338	0.042895	0.050193

Table 12: Protein Content (Lowry et al) of *Hymenophyllaceae* Species & Sun Ferns

SPECIES	R	WEIGHT (G)	A500	PROTEIN (MG/G)	PROT/CHL
H. denticulatum	1	0.035	0.232	8.85	1.49
	2	0.034	0.244	11.5	1.81
	3	0.032	0.223	7.5	1.33
	4	0.036	0.24	10.32	1.86
	5	0.032	0.235	10.4	1.92
			MEAN	9.714	1.682
			SD	1.555307044	0.257623757
			SE	0.695554455	0.115212847
T. meiofolium	1	0.103	0.526	5.02	0.65
	2	0.12	0.54	4.49	0.5
	3	0.137	0.55	4.05	0.5
	4	0.126	0.583	4.8	0.54
	5	0.11	0.506	4.42	0.47
			MEAN	4.556	0.532
			SD	0.372061823	0.070498227
			SE	0.166391106	0.031527766
H. acanthoides	1	0.052	0.409	6.46	1.11
	2	0.035	0.468	10.58	2.07
	3	0.041	0.476	8.62	5.79
	4	0.032	0.508	9.41	2.43
	5	0.039	0.437	7.74	2.98
			MEAN	8.562	2.876
			SD	1.572329482	1.765610376
			SE	0.703167121	0.789604965
H. serrulatum	1	0.104	2.083	56.29	12.03
	2	0.109	2.387	62.35	12.55
	3	0.103	2.016	54.83	4.37
	4	0.131	2.281	49.37	15.38
	5	0.1	1.53	41.42	6.73

			MEAN	52.852	10.212
			SD	7.886052244	4.520311936
			SE	3.526749778	2.021544954
H. javanicum	1	0.558	0.679	1.35	0.24
	2	0.5	0.609	1.29	0.26
	3	0.52	0.646	1.35	0.36
	4	0.54	0.549	1.02	0.21
	5	0.55	0.693	1.2	0.23
			MEAN	1.242	0.26
			SD	0.138455769	0.058736701
			SE	0.061919302	0.026267851
C. obscorum	1	0.54	0.625	1.24	0.155
	2	0.53	0.63	1.28	0.22
	3	0.525	0.645	1.34	0.21
	4	0.55	0.632	1.24	0.18
	5	0.532	0.628	1.27	0.18
			MEAN	1.274	0.189
			SD	0.040987803	0.02607681
			SE	0.018330303	0.011661904
H. exsertum	1	0.31	0.308	0.29	0.1
	2	0.33	0.434	0.57	0.15
	3	0.31	0.326	0.33	0.12
	4	0.31	0.315	0.31	0.1
	5	0.32	0.308	0.28	0.06
			MEAN	0.356	0.106
			SD	0.12116105	0.032863353
			SE	0.054184869	0.014696938
H. blandum	1	0.3	0.296	0.27	0.06
	2	0.31	0.289	0.24	0.05
	3	0.3	0.282	0.22	0.08
	4	0.3	0.264	0.19	0.08
	5	0.32	0.34	0.36	0.16
			MEAN	0.256	0.086

			SD	0.06503845	0.043358967
			SE	0.029086079	0.019390719
SUN					
Dicranopteris	1	0.052	0.646	13.52	5.24
linearis	2	0.051	0.625	13.14	5.33
	3	0.054	0.66	13.42	4.7
	4	0.055	0.672	13.51	7.29
	5	0.05	0.669	14.77	4.74
			MEAN	13.672	5.46
			SD	0.632747975	1.061861573
			SE	0.282973497	0.474878932
Nephrolepis	1	0.051	0.585	11.7	7.68
biserrata	2	0.053	0.611	12.24	8.1
	3	0.054	0.59	11.41	8.34
	4	0.052	0.62	12.74	8.05
	5	0.051	0.605	12.54	5.6
			MEAN	12.126	7.554
			SD	0.560249944	1.117577738
			SE	0.250551392	0.499795958

Table 13: Protein Content (Bradford et al) of *Hymenophyllaceae* Species & Sun Ferns

SPECIES	R	WEIGHT (G)	A500	PROTEIN (MG/G)	PROT/CHL
H. denticulatum	1	0.066	0.24	4.62	0.78
	2	0.06	0.252	5.35	0.84
	3	0.062	0.27	5.55	0.98
	4	0.061	0.253	5.29	0.95
	5	0.06	0.22	4.68	0.87
			MEAN	5.098	0.884
			SD	0.420678024	0.081424812
			SE	0.188132932	0.036414283
T. meiofolium	1	0.0501	0.226	5.75	0.74
	2	0.0521	0.224	5.48	0.61
	3	0.051	0.219	5.48	0.67
	4	0.0509	0.224	5.61	0.64
	5	0.0521	0.228	5.58	0.6
			MEAN	5.58	0.652
			SD	0.111579568	0.056302753
			SE	0.0498999	0.025179357
H. acanthoides	1	0.0586	0.241	5.25	0.9
	2	0.055	0.247	5.73	1.12
	3	0.056	0.228	5.19	3.48
	4	0.053	0.241	5.8	1.49
	5	0.0575	0.243	5.39	2.07
			MEAN	5.472	1.812
			SD	0.278244497	1.032361371
			SE	0.124434722	0.461686041
H. serrulatum	1	0.052	0.765	18.8	4.02
	2	0.055	0.715	16.6	3.34
	3	0.056	0.695	18.1	1.44
	4	0.053	0.708	16.1	5.02
	5	0.0575	0.68	17.3	2.81

			MEAN	17.38	3.326
			SD	1.094074952	1.33928339
			SE	0.489285193	0.59894574
H. javanicum	1	0.1	0.21	2.68	0.48
	2	0.1	0.245	3.11	0.63
	3	0.1	0.222	2.77	0.97
	4	0.1	0.194	2.47	0.51
	5	0.1	0.266	3.33	0.65
			MEAN	2.872	0.648
			SD	0.344702771	0.194473649
			SE	0.154155765	0.08697126
C. obscorum	1	0.0576	0.235	5.2	0.65
	2	0.0554	0.237	5.25	0.91
	3	0.0561	0.205	4.66	0.74
	4	0.0541	0.215	5.07	0.74
	5	0.0522	0.216	5.28	0.76
			MEAN	5.092	0.76
			SD	0.254499509	0.094074439
			SE	0.11381564	0.042071368
H. exsertum	1	0.1	0.146	1.83	0.61
	2	0.1	0.14	1.76	0.46
	3	0.11	0.133	1.59	0.56
	4	0.11	0.149	1.79	0.59
	5	0.1	0.169	2.13	0.44
			MEAN	1.82	0.532
			SD	0.195959179	0.077265775
			SE	0.087635609	0.034554305
H. blandum	1	0.1	0.162	2.04	0.43
	2	0.11	0.122	1.43	0.33
	3	0.1	0.134	1.62	0.59
	4	0.11	0.121	1.43	0.58

	5	0.11	0.124	1.48	0.66
			MEAN	1.6	0.518
			SD	0.257972867	0.1344247
			SE	0.115368973	0.060116553
SUN					
Dicranopteris	1	0.05	0.585	14.75	5.71
linearis	2	0.05	0.524	13.08	5.31
	3	0.05	0.51	12.63	4.43
	4	0.05	0.521	13.24	7.15
	5	0.05	0.514	12.91	4.15
			MEAN	13.322	5.35
			SD	0.829620395	1.1892855
			SE	0.37101752	0.531864644
Nephrolepis	1	0.05	0.811	20.16	13.22
biserrata	2	0.05	0.715	17.37	11.49
	3	0.05	0.819	20.48	14.97
	4	0.05	0.856	21.37	13.5
	5	0.05	0.813	19.94	8.91
			MEAN	19.864	12.418
			SD	1.49673979	2.318074632
			SE	0.669362383	1.036674491

Table 14: Protein Content (BCA Assay) Of *Hymenophyllaceae* Species & Sun Ferns

SPECIES	R	WEIGHT (G)	A500	PROTEIN (MG/G)	PROT/CHL
H. denticulatum	1	0.0662	0.701	1.5	0.25
	2	0.0601	0.677	1.45	0.23
	3	0.062	0.704	1.46	0.26
	4	0.061	0.738	1.55	0.28
	5	0.06	0.718	1.54	0.28
			MEAN	1.5	0.26
			SD	0.045276926	0.021213203
			SE	0.020248457	0.009486833
T. meiofolium	1	0.1089	0.343	4.05	0.52
	2	0.1098	0.339	3.97	0.44
	3	0.1216	0.412	4.35	0.53
	4	0.1082	0.324	3.85	0.44
	5	0.111	0.312	3.61	0.39
			MEAN	3.966	0.464
			SD	0.271440601	0.059413803
			SE	0.121391927	0.026570661
H. acanthoides	1	0.0055	0.207	4.84	0.83
	2	0.003	0.217	9.29	1.82
	3	0.0029	0.183	8.11	5.44
	4	0.0031	0.207	8.58	2.21
	5	0.0028	0.245	11.21	4.32
			MEAN	8.406	2.924
			SD	2.317224633	1.896873744
			SE	1.03629436	0.848307727
H. serrulatum	1	0.0037	1.054	36.61	7.82
	2	0.0041	1.074	33.66	6.77
	3	0.0036	0.904	32.27	2.57
	4	0.0059	1.546	33.67	7.37
	5	0.004	0.991	31.84	5.18

			MEAN	33.61	5.942
			SD	1.866453857	2.133229945
			SE	0.83470354	0.954009434
H. javanicum	1	0.055	0.343	8.01	1.45
	2	0.0535	0.54	13	2.62
	3	0.054	0.45	10.7	3.73
	4	0.0525	0.389	9.5	1.98
	5	0.0515	0.412	10.3	2
			MEAN	10.302	2.356
			SD	1.825792978	0.872714157
			SE	0.816519443	0.390289636
C. obscorum	1	0.0576	0.15	3.35	0.42
	2	0.0554	0.098	2.27	0.39
	3	0.0561	0.113	2.59	0.41
	4	0.0541	0.14	3.33	0.49
	5	0.0522	0.136	3.35	0.48
			MEAN	2.978	0.438
			SD	0.512952239	0.044384682
			SE	0.229399215	0.019849433
H. exsertum	1	0.0049	0.153	4.01	1.33
	2	0.0046	0.152	4.25	1.11
	3	0.0043	0.143	4.27	1.51
	4	0.0044	0.157	4.59	1.51
	5	0.0035	0.16	5.87	1.21
			MEAN	4.598	1.334
			SD	0.740351268	0.178549713
			SE	0.331095152	0.079849859
H. blandum	1	0.005	0.167	4.29	0.9
	2	0.0051	0.158	3.98	0.91
	3	0.0049	0.149	3.91	1.43
	4	0.0052	0.162	4	1.63

	5	0.0048	0.165	4.42	1.96
			MEAN	4.12	1.366
			SD	0.22192341	0.461443388
			SE	0.099247166	0.206363757
SUN					
Dricapnoteris	1	0.0501	3.465	17.77	6.88
linearis	2	0.0519	3.324	16.46	6.68
	3	0.0522	3.831	18.86	6.61
	4	0.0509	3.852	19.45	10.5
	5	0.0508	3.53	17.86	5.74
			MEAN	18.08	7.282
			SD	1.14610209	1.851275236
			SE	0.512552436	0.827915455
Nephrolepis	1	0.049	2.427	12.73	8.35
biserrata	2	0.0487	2.421	12.78	8.45
	3	0.0539	2.437	11.62	8.49
	4	0.0495	2.465	12.8	8.09
	5	0.0512	2.432	12.26	5.48
			MEAN	12.438	7.772
			SD	0.508448621	1.290705234
			SE	0.227385136	0.577220928

Table 15: BSA Standard Curve (Bradford Assay)

[BSA], MG/ML	VOL BSA	VOL BUFFER		A595			
			R1	R2	R3	AVERAGE	SD
0	0	100 UL	0	0	0	0	0
0.2	20	80	0.083	0.096	0.104	0.094333	0.010599
0.4	40	60	0.208	0.229	0.187	0.208	0.021
0.6	60	40	0.242	0.309	0.257	0.269333	0.035162
0.8	80	20	0.305	0.322	0.324	0.317	0.01044
1	100	0	0.356	0.356	0.327	0.346333	0.016743

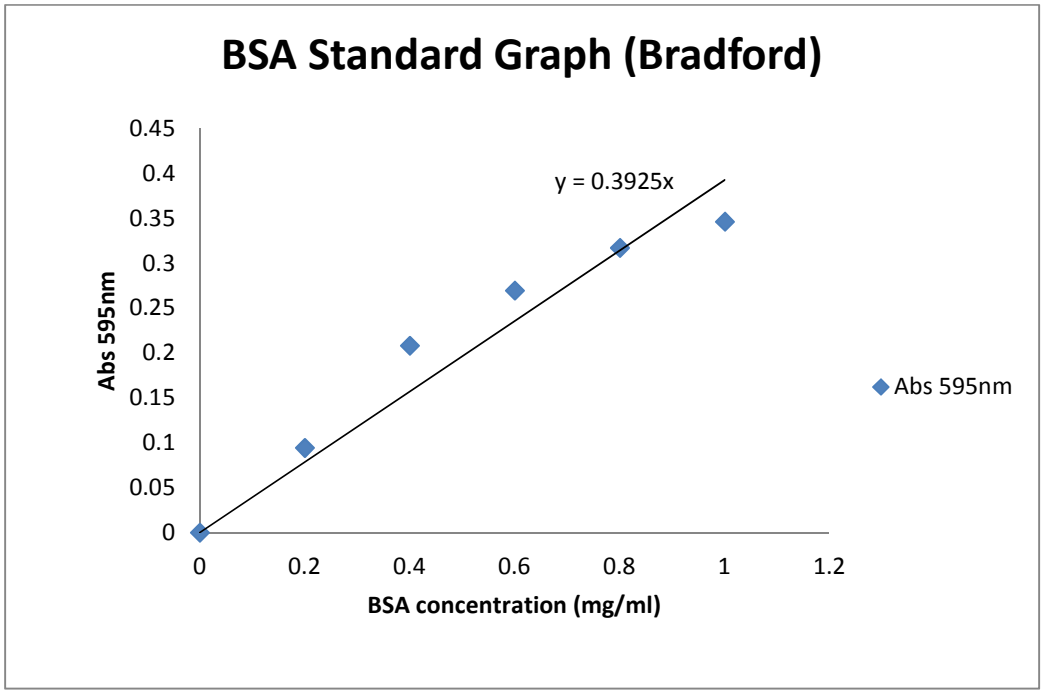


Table 16: BSA Standard Curve (BCA Assay)

[BSA],UG/ML		A562		AVERAGE	SD
	R1	R2	R3		
0	0	0	0	0	0
25	0.247	0.22	0.21	0.22566667	0.01914
125	0.93	0.964	0.935	0.943	0.018358
250	1.229	1.033	1.006	1.08933333	0.121706
500	2.259	2.114	2.063	2.14533333	0.101687
1000	3.8	3.655	3.65	3.70166667	0.085196

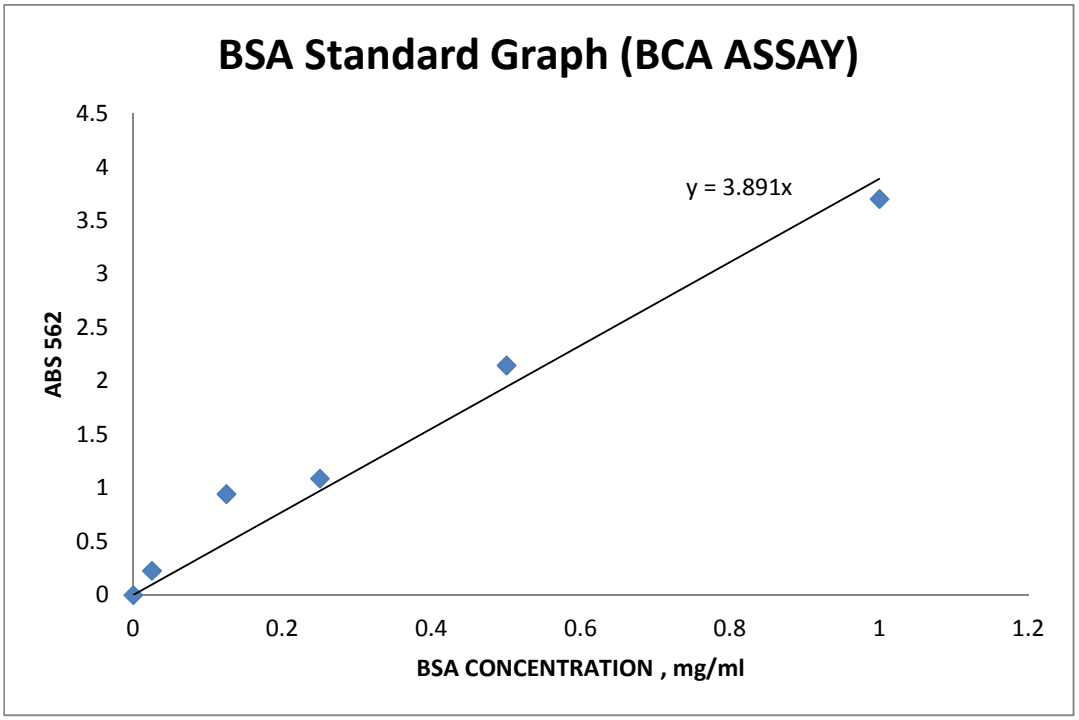


Table 17: BSA Standard Curve (Lowry assay)

[BSA],MG/ML		A500		AVERAGE	SD
	R1	R2	R3		
0.25	0.326	0.326	0.306	0.319333	0.011547
0.5	0.473	0.522	0.585	0.526667	0.056146
0.75	0.713	0.675	0.676	0.688	0.021656
1	0.844	0.83	0.833	0.835667	0.007371
1.5	1.105	1.36	1.233	1.232667	0.1275
2	1.351	1.425	1.52	1.432	0.084717

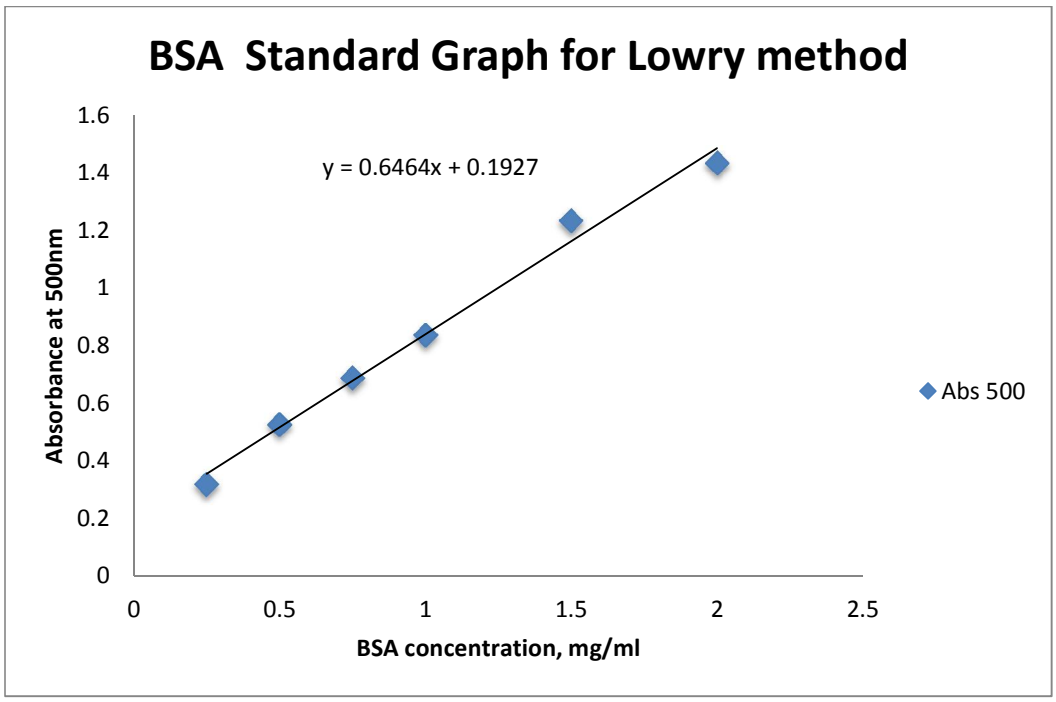


Table 18: Chloroplast Number Per Cell Profile (N=20) of Eight *Hymenophyllaceae* Species

REPLICATES	*H. acanthoides*	*H. blandum*	*H. serrulatum*	*H. exsertum*	*Javanicum*	*H. denticulatum*	*H. obscorum*
1	52	29	57	66	36	33	151
2	71	40	65	44	34	43	128
3	59	35	60	42	50	33	130
4	71	33	56	47	36	42	137
5	63	39	75	43	45	37	108
6	39	23	66	51	58	36	164
7	53	36	32	54	29	46	125
8	63	31	43	53	57	48	133
9	51	29	45	37	55	39	160
10	53	34	50	39	37	50	150
11	70	42	52	38	30	43	150
12	85	42	46	45	41	40	130
13	45	32	64	51	32	47	137
14	99	32	64	44	57	45	138
15	62	39	60	50	51	43	117
16	83	25	39	44	43	42	142
17	57	39	57	48	42	44	131
18	63	35	39	36	40	46	129
19	61	35	45	41	43	46	144
20	63	33	55	46	42	44	167
MEAN	63.15	34.15	53.5	45.95	42.9	42.35	138.55
SD	14.10216482	5.214100316	10.99521427	7.037306602	9.158832378	4.738143096	15.21590235
SE	3.153339917	1.165908275	2.458604653	1.573589594	2.047977179	1.059481005	3.402379199

Table 19: Chloroplast Size (µm) Of Eight *Hymenophyllaceae* Species

REPLICATES	*H. acanthoides*	*H. blandum*	*H. exsertum*	*H. serrulatum*	*H. javancium*	*H. denticulatum*	*H. obcsorum*
1	6.881	6.258	4.397	5.423	5.782	6.7	5.743
2	4.242	6.943	4.905	6.26	5.887	5.961	5.188
3	4.228	6.818	6.258	6.937	6.324	6.781	5.174
4	3.981	6.098	6.267	5.074	5.492	5.961	5.07
5	4.567	5.077	4.57	5.21	5.368	7.216	5.382
6	5.243	4.908	5.454	5.174	6.157	7.345	5.188
7	5.826	6.598	6.427	4.228	6.567	7.689	4.594
8	7.154	6.258	6.535	5.254	5.544	6.676	4.842
9	4.397	6.258	6.089	5.751	5.481	6.68	4.82
10	4.9	5.673	5.119	6.098	7.784	7.487	3.853
11	4.951	6.767	4.554	5.268	5.195	6.442	5.39
12	5.751	4.567	5.92	6.267	6.338	7.294	4.43
13	4.746	6.784	6.436	3.923	5.937	7.426	5.091
14	5.246	6.598	5.046	6.974	6.063	6.594	4.998
15	4.567	5.246	7.43	7.949	5.754	5.504	3.409
16	5.368	5.34	5.243	4.579	6.315	6.512	4.95
17	5.412	7.618	6.147	5.478	6.061	5.234	5.13
18	5.92	5.077	4.763	6.934	5.485	5.679	5.038
19	5.89	6.568	6.112	5.423	5.225	6.305	5.372
20	4.579	8.331	6.173	4.718	6.616	8.802	3.834
21	6.126	4.736	6.089	5.673	5.42	6.621	3.547
22	6.278	7.762	6.818	5.074	5.298	5.967	5.161

23	5.773	8.93	6.203	6.26	5.497	6.199	4.616
24	4.977	7.373	4.905	5.478	4.472	7.153	4.018
25	4.9	6.482	6.091	8.118	5.092	7.267	4.166
26	5.751	6.258	5.077	5.085	6.535	6.594	4.733
27	7.49	7.459	5.423	7.263	5.27	6.621	4.168
28	4.29	6.818	5.251	7.73	5.801	8.802	4.356
29	6.535	7.949	5.243	5.922	5.059	6.24	4.712
30	6.818	8.564	5.088	5.592	5.27	6.13	3.853
31	4.29	7.343	6.943	7.797	6.002	5.889	4.548
32	5.412	5.958	4.228	6.258	5.696	5.961	4.172
33	6.765	6.447	6.765	6.934	5.27	6.812	4.49
34	5.09	6.581	3.89	7.281	5.237	4.225	5.096
35	4.232	5.415	5.246	6.436	5.064	5.868	4.636
36	5.54	7.273	5.812	5.415	6.656	6.804	5.124
37	4.059	8.024	5.958	5.871	5.195	6.835	4.918
38	5.751	5.673	6.779	5.34	4.216	5.291	4.47
39	7.067	5.751	4.242	4.905	5.481	6.036	5.186
40	6.285	6.702	4.579	5.243	6.838	6.609	5.159
41	4.41	5.243	5.478	4.763	6.157	6.613	4.668
42	6.463	6.65	6.267	5.949	6.538	5.661	3.902
43	7.671	7.641	5.243	8.274	6.416	6.224	5.452
44	5.478	4.931	6.482	6.382	4.642	5.841	4.162
45	5.604	8.457	5.929	6.967	5.902	5.75	4.329
46	5.077	7.956	6.11	9.118	4.861	5.881	5.135
47	6.044	5.268	6.447	5.119	6.117	5.767	5.186
48	6.258	5.929	6.616	7.106	5.794	5.184	4.822

49	7.343	6.765	4.905	7.273	6.234	5.055	4.718
50	6.596	5.751	5.243	5.243	5.308	6.163	6.234
51	4.579	7.45	4.228	8.411	5.167	5.75	5.601
52	8.397	5.929	6.596	5.604	6.061	6.362	4.523
53	5.753	4.513	7.442	5.584	5.059	5.497	4.906
54	6.091	7.275	7.136	7.384	6.26	7.186	4.212
55	5.753	4.513	6.616	5.243	6.234	6.213	4.912
mean	5.614454545	6.464618182	5.731145455	6.091218182	5.736254545	6.388345455	4.752490909
SD	1.030880239	1.122093868	0.883631992	1.153470636	0.651922178	0.836035468	0.565507896
SE	0.139003863	0.151303106	0.119148913	0.155533949	0.087905169	0.112731	0.076253069

Table 20: Chlorophyll Fluorescence In Four Species Of *Hymenophyllaceae*

	R	1	2	3	4	5	Mean	SD	SE
Hymenophyllum serrulatum	Fo	454	706	491	437	476	512.8	109.953	49.1726
	Fm	1937	2397	1964	1259	2258	1963	439.225	196.427
	Fv/Fm	0.766	0.705	0.75	0.653	0.789	0.7326	0.05408	0.02418
Hymenophyllum javanicum	Fo	473	576	574	715	505	568.6	93.1413	42
	Fm	2169	2254	2132	2459	2009	2204.6	167.3	75
	Fv/Fm	0.782	0.744	0.731	0.709	0.749	0.743	0.02673	0.01
Hymenophyllum denticulatum	Fo	318	427	276	377	497	379	87.41	39
	Fm	1673	2258	1567	2009	2269	1955.2	325.342	145
	Fv/Fm	0.81	0.811	0.824	0.812	0.781	0.8076	0.01592	0.009
Trichomanes meiofolium	Fo	488	462	463	595	544	510.4	57.8299	26
	Fm	2522	1949	2219	2432	2016	2227.6	250.458	112
	Fm/Fv	0.807	0.763	0.791	0.755	0.73	0.7692	0.03033	0.01

Table 21: Photosynthetic rate of eight *Hymenophyllaceae* Species

A) Hyemnophyllum denticulatum

Light Irradiance	Photo out R1	Photo out R2	Photo out R3	MEAN	SD	SE
0	-2.00527	2.480604	-2.6907	-0.73846	2.808776	1.6216473
15	2.383268	2.814737	2.773039	2.657014	0.237986	0.1374016
30	3.129661	2.662806	3.661108	3.151192	0.499499	0.288386
60	2.059525	4.221316	2.595252	2.958698	1.125791	0.6499756
90	2.823777	7.995371	2.823777	4.547642	2.985821	1.7238645
120	4.089011	8.749139	4.970237	5.936129	2.475663	1.4293247
150	10.63518	8.755077	10.82745	10.07257	1.145026	0.6610812
300	10.98895	10.75728	10.98895	10.91172	0.133757	0.0772244
600	10.01757	11.03639	11.74463	10.93286	0.86817	0.5012384
900	11.00021	11.21474	10.01757	10.74418	0.638335	0.3685428
1200	11.97748	11.48127	11.74813	11.73563	0.248343	0.1433807
1500	12.82982	12.52571	12.98248	12.77934	0.232529	0.1342509
2000	12.60773	13.74422	12.60773	12.98656	0.656154	0.3788306

B) Hymenophyllum javanicum

Light Irradiance	Photo out R1	Photo out R2	Photo out R3	mean	SD	SE
0	1.308687	1.831183	1.052091	1.39732	0.397037	0.2292292
15	1.605209	1.095521	2.487661	1.729464	0.704339	0.40665
30	2.619467	1.831605	2.986195	2.479089	0.589957	0.3406116
60	3.521996	2.859781	2.541763	2.974513	0.500087	0.2887254
90	4.752535	2.985541	6.422868	4.720315	1.71889	0.9924018
120	5.089907	3.210522	6.879102	5.059844	1.834475	1.0591344
150	5.415071	3.265952	6.759293	5.146772	1.762058	1.0173244
300	5.667363	3.286052	7.59213	5.515182	2.157069	1.2453841
600	5.600927	3.210522	6.174896	4.995448	1.572206	0.9077136
900	5.199904	4.802588	6.163488	5.38866	0.69981	0.4040354
1200	5.286052	4.985541	6.684427	5.652007	0.90664	0.5234486
1500	5.010562	4.859781	6.094379	5.321574	0.673502	0.3888464
2000	5.914481	5.210522	6.933097	6.019367	0.866064	0.5000222

C) Hymenophyllum serrulatum

Light Irradiance	Photo out	Photo out	Photo out			
	R1	R2	R3	MEAN	SD	SE
0	1.446849	1.538684	1.019151	1.334895	0.277271	0.1600823
15	3.423735	6.830267	4.13098	4.794994	1.797721	1.0379148
30	8.787241	6.619083	9.30632	8.237548	1.425459	0.8229889
60	7.118928	7.980116	7.580754	7.559933	0.430971	0.2488213
90	7.416073	6.496324	7.023575	6.978657	0.461517	0.266457
120	9.345372	8.404122	7.48438	8.411291	0.930516	0.5372339
150	9.12591	9.790338	9.123826	9.346691	0.384211	0.2218242
300	12.0304	8.112207	9.050173	9.730927	2.045882	1.1811904
600	12.68339	8.63171	9.926703	10.41393	2.069315	1.1947194
900	12.72683	11.15695	10.17205	11.35195	1.288505	0.7439186
1200	12.9385	8.745649	9.765588	10.48324	2.186611	1.2624402
1500	13.50701	10.32941	9.567531	11.13465	2.089544	1.2063987
2000	9.823218	7.725256	9.092113	8.880196	1.064915	0.6148287

D) Trichomanes meiofolium

LIGHT IRRADIANCE	Photo out	Photo out	Photo out			
	R1	R2	R3	MEAN	SD	SE
0	1.494635	3.546946	2.778546	2.606709	1.03689	0.5986489
15	4.494643	5.902561	3.564926	4.654043	1.176941	0.6795073
30	3.465272	8.861652	5.406937	5.911287	2.733314	1.5780798
60	5.401827	6.223328	8.425705	6.68362	1.563605	0.9027479
90	10.52088	11.35078	9.204048	10.35857	1.082529	0.6249982
120	8.020567	11.61241	12.18195	10.60498	2.256208	1.3026222
150	10.78985	11.39263	12.65174	11.61141	0.950033	0.5485019
300	10.97448	9.371065	12.25439	10.86664	1.444683	0.8340884
600	10.43799	12.82203	13.84377	12.36793	1.747712	1.009042
900	9.293232	13.2369	11.32436	11.28483	1.972133	1.1386116
1200	10.31962	10.37995	11.87771	10.85909	0.882664	0.5096063
1500	8.297494	11.97308	11.92181	10.73079	2.107455	1.2167399
2000	7.259622	11.18253	11.20054	9.8809	2.270111	1.3106494

E) Hymenophyllum exsertum

LIIGHT	Photo	Photo	Photo			
IRRADIANCE	out	out	out			
	R1	R2	R3	MEAN	SD	SE
0	3.385552	3.892294	4.32976	3.869202	0.472527	0.2728138
15	3.61549	2.532948	3.972118	3.373519	0.749476	0.4327104
30	5.232603	5.033635	6.876802	5.714346	1.011619	0.5840587
60	6.009569	6.294836	5.572484	5.958963	0.363825	0.2100546
90	10.07881	12.85588	11.07798	11.33756	1.406614	0.8121087
120	16.20102	14.39379	13.39594	14.66358	1.42187	0.820917
150	12.96072	15.53525	13.44983	13.98193	1.367259	0.7893875
300	12.16606	13.02196	14.30368	13.1639	1.075857	0.6211461
600	15.49572	14.70544	14.84096	15.01404	0.422617	0.2439979
900	15.97027	16.80613	13.85209	15.54283	1.522704	0.8791337
1200	13.52111	12.98816	14.77169	13.76032	0.91551	0.5285697
1500	15.70396	13.63832	10.99487	13.44572	2.360444	1.3628028
2000	15.06309	13.06549	15.07022	14.3996	1.15538	0.6670587

F) Hymenophyllum blandum

LIGHT	Photo	Photo	Photo			
IRRADIANCE	out	out	out			
	R1	R2	R3	MEAN	SD	SE
0	0.122456	0.982346	0.457689	0.52083	0.433408	0.6583374
15	0.024563	0.123547	-1.23568	-0.36252	0.757793	0.8705128
30	0.805268	0.453787	0.805268	0.688108	0.202928	0.4504752
60	0.824585	1.009674	0.824585	0.886281	0.106861	0.3268966
90	0.830613	1.238766	1.67685	1.248743	0.423207	0.6505434
120	0.888092	1.987457	1.888092	1.58788	0.608068	0.7797868
150	1.17578	1.908548	1.117578	1.400635	0.440827	0.6639478
300	3.271418	3.412781	2.096745	2.926981	0.722471	0.8499832
600	2.541188	4.112058	2.541188	3.064811	0.906942	0.9523351
900	2.92807	4.512347	2.929077	3.456498	0.914392	0.9562386
1200	3.709942	4.567289	2.708794	3.662008	0.930174	0.9644554
1500	2.825993	4.221358	3.87826	3.64187	0.727098	0.8527002
2000	3.548472	3.334786	3.544577	3.475945	0.122263	0.349661

G) Hymenophyllum acanthoides

LIGHT	Photo	Photo	Photo			
IRRADIANCE	out	out	out			
	R1	R2	R3	MEAN	SD	SE
0	0.968359	0.566736	0.415071	0.650055	0.2859	0.5346957
15	0.75251	0.415071	0.968359	0.71198	0.278862	0.5280738
30	3.566736	3.968359	5.667363	4.40082	1.11509	1.055978
60	4.150708	4.752501	5.600927	4.834712	0.728596	0.8535784
90	4.830105	5.830105	6.991984	5.884064	1.081949	1.0401679
120	7.29247	9.246992	8.301045	8.280169	0.977428	0.9886497
150	7.856009	9.266139	8.704561	8.608903	0.709915	0.8425644
300	12.87046	12.87046	14.75254	13.49782	1.086619	1.0424101
600	15.08991	15.89907	15.08991	15.35963	0.467171	0.6834994
900	15.57791	16.57037	16.57794	16.24208	0.575197	0.7584172
1200	16.57037	16.57794	15.70373	16.28402	0.502554	0.7089102
1500	16.63769	16.37694	14.63769	15.88411	1.087272	1.0427235
2000	16.99566	15.65574	16.55266	16.40135	0.682653	0.8262285

H) Cephalomanes obscorum

LIGHT	Photo	Photo	Photo			
IRRADIANCE	out	out	out			
	R1	R2	R3	MEAN	SD	SE
0	0.676451	0.764509	0.067645	0.502868	0.379477	0.6160172
15	6.883425	6.168834	6.168834	6.407031	0.412569	0.6423156
30	13.3986	10.39967	10.39967	11.39931	1.731435	1.3158399
60	13.69381	10.69302	10.69302	11.69328	1.732508	1.3162478
90	15.72028	15.66322	15.66322	15.68224	0.032944	0.1815045
120	15.06115	15.81153	15.86061	15.57777	0.448072	0.6693817
150	17.63709	16.29834	16.29834	16.74459	0.772926	0.8791621
300	17.65836	16.41003	16.41003	16.82614	0.720726	0.848956
600	17.95831	18.27675	18.27675	18.1706	0.18385	0.4287774
900	20.38061	19.31035	19.31035	19.6671	0.617917	0.7860769
1200	20.76767	20.38061	20.38061	20.50963	0.223468	0.4727241
1500	20.57757	20.76767	20.76767	20.7043	0.109756	0.3312938
2000	21.74551	20.77767	20.77767	21.10029	0.55878	0.747516

Table 22: Photosynthetic rate of Sun Ferns Species

A) Dicranopteris linearis

	Photo	Photo	Photo			
LIGHT	out	out	out			
IRRADIANCE	R1	R2	R3	MEAN	SD	SE
0	2.59395	2.113007	4.740116	3.149024	1.398752	0.80757
15	3.480666	3.514695	5.863824	4.286395	1.3662	0.788776
30	9.260699	3.191877	7.630674	6.694416	3.140873	1.813384
60	10.92339	4.327287	7.154615	7.468432	3.309232	1.910586
90	13.84874	13.38819	8.012873	11.74993	3.244573	1.873255
120	14.75333	15.24003	8.830939	12.94143	3.5681	2.060043
150	16.02256	16.37648	11.51023	14.63642	2.713141	1.566433
300	19.01339	18.5739	12.59227	16.72652	3.587105	2.071016
600	21.5586	20.08622	16.91981	19.52154	2.370388	1.368544
900	23.03109	24.90836	18.17478	22.03808	3.474887	2.006227
1200	24.34633	24.95236	17.34092	22.2132	4.230389	2.442416
1500	24.89489	24.51317	17.52364	22.31057	4.149994	2.396
2000	24.60896	23.14282	15.53731	21.09637	4.869773	2.811565

B) Nephrolepis biserrata

	Photo	Photo	Photo			
LIGHT	out	out	out			
IRRADIANCE	R1	R2	R3	MEAN	SD	SE
0	1.471945	1.733182	2.845284	2.016804	0.729277	0.421048
15	1.890971	2.873409	3.936869	2.900416	1.023217	0.590754
30	7.480154	2.220851	3.635362	4.445456	2.721627	1.571332
60	6.968312	4.354814	3.085221	4.802783	1.979926	1.143111
90	9.728861	8.226915	4.596467	7.517414	2.638733	1.523473
120	8.677347	10.41679	10.47475	9.856296	1.021411	0.589712
150	9.850653	13.20619	17.09635	13.3844	3.626135	2.09355
300	14.34273	23.80661	25.19254	21.11396	5.904861	3.409173
600	26.58666	24.92563	34.55176	28.68802	5.14562	2.970825
900	27.38341	29.80611	33.38114	30.19022	3.017257	1.742014
1200	29.46385	24.22906	39.57008	31.08767	7.798355	4.502382
1500	29.8413	25.49247	38.04193	31.12523	6.372491	3.67916
2000	29.1629	28.68388	26.90136	28.24938	1.191733	0.688047